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(57) Abstract

Isolated and purified nucleic acid molecules that encode a luciferase from *Renilla mulleri*, *Gaussia* and *Pleuromamma*, and the proteins encoded thereby are provided. Isolated and purified nucleic acids encoding green fluorescent proteins from the genus *Renilla* and *Ptilosarcus*, and the green fluorescent proteins encoded thereby are also provided. Compositions and combinations comprising the green fluorescent proteins and/or the luciferase are further provided.

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**LUCIFERASES, FLUORESCENT PROTEINS, NUCLEIC ACIDS ENCODING THE
LUCIFERASES AND FLUORESCENT PROTEINS AND THE USE THEREOF IN
DIAGNOSTICS, HIGH THROUGHPUT SCREENING AND NOVELTY ITEMS**

RELATED APPLICATIONS

- 5 This application claims priority to U.S. provisional application Serial No. 60/102,939, filed October 1, 1998, to Bruce Bryan and Christopher Szent-Gyorgyi, entitled "LUCIFERASES, FLUORESCENT PROTEINS, NUCLEIC ACIDS ENCODING THE LUCIFERASES AND FLUORESCENT PROTEINS AND THE USE THEREOF IN DIAGNOSTICS, HIGH THROUGHPUT SCREENING AND NOVELTY
10 ITEMS". Priority is also claimed to U.S. provisional application Serial No. 60/089,367, filed June 15, 1998, to Bruce Bryan and Christopher Szent-Gyorgyi, entitled "GAUSSIA LUCIFERASE, NUCLEIC ACIDS ENCODING THE LUCIFERASE AND METHODS USING THE LUCIFERASE", and to U.S. provisional application Serial No. 60/079,624, filed March 27, 1998, to Bruce
15 Bryan and Christopher Szent-Gyorgyi, entitled "RENILLA GREEN FLUORESCENT PROTEIN COMPOSITIONS AND METHODS." For U.S. purposes, benefit of priority to each of these applications is claimed under 35 U.S.C. § 119(e).

- This application is also related to subject matter in U.S. application Serial No. 08/757,046, filed November 25, 1996, to Bruce Bryan entitled
20 "BIOLUMINESCENT NOVELTY ITEMS", now U.S. Patent No. 5,876,995, issued March 2, 1999, and in U.S. application Serial No. 08/597,274, filed February 6, 1996, to Bruce Bryan, entitled "BIOLUMINESCENT NOVELTY ITEMS". This application is also related to U.S. application Serial No. 08/908,909, filed August 8, 1997, to Bruce Bryan entitled "DETECTION AND VISUALIZATION OF
25 NEOPLASTIC TISSUE AND OTHER TISSUES". The application is also related to U.S. application Serial No. 08/990,103, filed December 12, 1997, to Bruce Bryan entitled "APPARATUS AND METHODS FOR DETECTING AND IDENTIFYING INFECTIOUS AGENTS".

- The subject matter of each of the above noted U.S. applications and
30 provisional applications is herein incorporated by reference in its entirety.

-2-

FIELD OF INVENTION

The present invention relates to isolated and purified nucleic acids and encoded proteins from the genera *Renilla*, *Gaussia*, *Philocarpus* and *Pleuromamma*. More particularly, nucleic acids encoding luciferase and
5 fluorescent proteins from species of these genera are provided.

BACKGROUND OF THE INVENTION

Luminescence is a phenomenon in which energy is specifically channeled to a molecule to produce an excited state. Return to a lower energy state is accompanied by release of a photon (hv). Luminescence includes fluorescence,
10 phosphorescence, chemiluminescence and bioluminescence. Bioluminescence is the process by which living organisms emit light that is visible to other organisms. Luminescence may be represented as follows:



15 where X^* is an electronically excited molecule and hv represents light emission upon return of X^* to a lower energy state. Where the luminescence is bioluminescence, creation of the excited state derives from an enzyme catalyzed reaction. The color of the emitted light in a bioluminescent (or chemiluminescent or other luminescent) reaction is characteristic of the excited
20 molecule, and is independent from its source of excitation and temperature.

An essential condition for bioluminescence is the use of molecular oxygen, either bound or free in the presence of a luciferase. Luciferases, are oxygenases, that act on a substrate, luciferin, in the presence of molecular oxygen and transform the substrate to an excited state. Upon return to a lower
25 energy level, energy is released in the form of light [for reviews see, e.g., McElroy et al. (1966) in *Molecular Architecture in Cell Physiology*, Hayashi et al., eds., Prentice-Hall, Inc., Englewood Cliffs, NJ, pp. 63-80; Ward et al., Chapter 7 in *Chemi-and Bioluminescence*, Burr, ed., Marcel Dekker, Inc. NY, pp.321-358; Hastings, J. W. in (1995) *Cell Physiology:Source Book*, N. Sperelakis (ed.), Academic Press, pp 665-681; *Luminescence, Narcosis and Life in the Deep Sea*, Johnson, Vantage Press, NY, see, esp. pp. 50-56].
30

-3-

Though rare overall, bioluminescence is more common in marine organisms than in terrestrial organisms. Bioluminescence has developed from as many as thirty evolutionarily distinct origins and, thus, is manifested in a variety of ways so that the biochemical and physiological mechanisms responsible for

5 bioluminescence in different organisms are distinct. Bioluminescent species span many genera and include microscopic organisms, such as bacteria [primarily marine bacteria including *Vibrio* species], fungi, algae and dinoflagellates, to marine organisms, including arthropods, mollusks, echinoderms, and chordates, and terrestrial organism including annelid worms

10 and insects.

Assays employing bioluminescence

During the past twenty years, high-sensitivity biochemical assays used in research and in medicine have increasingly employed luminescence and fluorescence rather than radioisotopes. This change has been driven partly by

15 the increasing expense of radioisotope disposal and partly by the need to find more rapid and convenient assay methods. More recently, the need to perform biochemical assays *in situ* in living cells and whole animals has driven researchers toward protein-based luminescence and fluorescence. The uses of firefly luciferase for ATP assays, aequorin and obelin as calcium reporters,

20 *Vargula* luciferase as a neurophysiological indicator, and the *Aequorea* green fluorescent protein as a protein tracer and pH indicator show the potential of bioluminescence-based methods in research laboratories.

Bioluminescence is also beginning to directly impact medicine and biotechnology; for example, *Aequorea* GFP is employed to mark cells in murine

25 model systems and as a reporter in high throughput drug screening. *Renilla* luciferase is under development for use in diagnostic platforms.

Bioluminescence generating systems

Bioluminescence, as well as other types of chemiluminescence, is used for quantitative determinations of specific substances in biology and medicine.

30 For example, luciferase genes have been cloned and exploited as reporter genes in numerous assays, for many purposes. Since the different luciferase systems have different specific requirements, they may be used to detect and quantify a

-4-

variety of substances. The majority of commercial bioluminescence applications are based on firefly [*Photinus pyralis*] luciferase. One of the first and still widely used assays involves the use of firefly luciferase to detect the presence of ATP. It is also used to detect and quantify other substrates or co-factors in the
5 reaction. Any reaction that produces or utilizes NAD(H), NADP(H) or long chain aldehyde, either directly or indirectly, can be coupled to the light-emitting reaction of bacterial luciferase.

Another luciferase system that has been used commercially for analytical purposes is the *Aequorin* system. The purified jellyfish photoprotein, aequorin,
10 is used to detect and quantify intracellular Ca^{2+} and its changes under various experimental conditions. The *Aequorin* photoprotein is relatively small [$\sim 20\text{kDa}$], nontoxic, and can be injected into cells in quantities adequate to detect calcium over a large concentration range [3×10^{-7} to 10^{-4} M].

Because of their analytical utility, luciferases and substrates have been
15 studied and well-characterized and are commercially available [e.g., firefly luciferase is available from Sigma, St. Louis, MO, and Boehringer Mannheim Biochemicals, Indianapolis, IN; recombinantly produced firefly luciferase and other reagents based on this gene or for use with this protein are available from Promega Corporation, Madison, WI; the aequorin photoprotein luciferase from
20 jellyfish and luciferase from *Renilla* are commercially available from Sealite Sciences, Bogart, GA; coelenterazine, the naturally-occurring substrate for these luciferases, is available from Molecular Probes, Eugene, OR]. These luciferases and related reagents are used as reagents for diagnostics, quality control, environmental testing and other such analyses.

25 Because of the utility of luciferases as reagents in analytical systems and the potential for use in high throughput screening systems, there is a need to identify and isolated a variety of luciferases that have improved or different spectral properties compared to those presently available. For all these reasons, it would be advantageous to have luciferases from a variety of species, such as
30 *Gaussia* and various *Renilla* species available.

Fluorescent Proteins

Reporter genes, when co-transfected into recipient cells with a gene of interest, provide a means to detect transfection and other events. Among reporter genes are those that encode fluorescent proteins. The
5 bioluminescence generating systems described herein are among those used as reporter genes. To increase the sensitivity bioluminescence generating systems have been combined with fluorescent compounds and proteins, such as naturally fluorescent phycobiliproteins. Also of interest are the fluorescent proteins that are present in a variety of marine invertebrates, such as the green
10 and blue fluorescent proteins, particularly the green fluorescent protein (GFP) of *Aequorea victoria*.

The green fluorescent proteins (GFP) constitute a class of chromoproteins found only among certain bioluminescent coelenterates. These accessory proteins are fluorescent and function as the ultimate bioluminescence
15 emitter in these organisms by accepting energy from enzyme-bound, excited-state oxyluciferin (e.g., see Ward *et al.* (1979) J. Biol. Chem. 254:781-788; Ward *et al.* (1978) Photochem. Photobiol. 27:389-396; Ward *et al.* (1982) Biochemistry 21:4535-4540).

The best characterized GFPs are those isolated from the jellyfish species
20 *Aequorea*, particularly *Aequorea victoria* (*A. victoria*) and *Aequorea forskålea* (Ward *et al.* (1982) Biochemistry 21:4535-4540; Prendergast *et al.* (1978) Biochemistry 17:3448-3453). Purified *A. victoria* GFP is a monomeric protein of about 27 Kda that absorbs blue light with excitation wavelength maximum of 395 nm, with a minor peak at 470 nm, and emits green fluorescence with an
25 emission wavelength of about 510 nm and a minor peak near 540 nm (Ward *et al.* (1979) Photochem. Photobiol. Rev 4:1-57). This GFP has certain limitations. The excitation maximum of the wildtype GFP is not within the range of wavelengths of standard fluorescein detection optics.

The detection of green fluorescence does not require any exogenous
30 substrates or co-factors. Instead, the high level of fluorescence results from the intrinsic chromophore of the protein. The chromophore includes modified amino acid residues within the polypeptide chain. For example, the fluorescent

-6-

chromophore of *A. victoria* GFP is encoded by the hexapeptide sequence, FSYGVO, encompassing amino acid residues 64-69. The chromophore is formed by the intramolecular cyclization of the polypeptide backbone at residues Ser65 and Gly67 and the oxidation of the α - β bond of residue Tyr66 (e.g., see
5 Cody *et al.* (1993) Biochemistry 32:1212-1218; Shimomura (1978) FEBS Letters 104:220-222; Ward *et al.* (1989) Photochem. Photobiol. 49:62S). The emission spectrum of the isolated chromophore and the denatured protein at neutral Ph do not match the spectrum of the native protein, suggesting that chromophore formation occurs post-translationally (e.g., see Cody *et al.* (1993)
10 Biochemistry 32:1212-1218).

In addition, the crystal structure of purified *A. victoria* GFP has been determined (e.g., see Ormö (1996) Science 273:1392-1395). The predominant structural features of the protein are an 11-stranded β barrel that forms a nearly perfect cylinder wrapping around a single central α -helix, which contains the
15 modified *p*-hydroxybenzylideneimidazolidinone chromophore. The chromophore is centrally located within the barrel structure and is completely shielded from exposure to bulk solvent.

DNA encoding an isotype of *A. victoria* GFP has been isolated and its nucleotide sequence has been determined (e.g., see Prasher (1992) Gene
20 111:229-233). The *A. victoria* CDNA contains a 714 nucleotide open reading frame that encodes a 238 amino acid polypeptide of a calculated M_r of 26,888 Da. Recombinantly expressed *A. victoria* GFPs retain their ability to fluoresce *in vivo* in a wide variety organisms, including bacteria (e.g., see Chalfie *et al.* (1994) Science 263:802-805; Miller *et al.* (1997) Gene 191:149-153), yeast
25 and fungi (Fey *et al.* (1995) Gene 165:127-130; Straight *et al.* (1996) Curr. Biol. 6:1599-1608; Cormack *et al.* (1997) Microbiology 143:303-311), *Drosophila* (e.g., see Wang *et al.* (1994) Nature 369:400-403; Plautz (1996) Gene 173:83-87), plants (Heinlein *et al.* (1995); Casper *et al.* (1996) Gene 173:69-73), fish (Amsterdam *et al.* (1995)), and mammals (Ikawa *et al.*
30 (1995). *Aequorea* GFP vectors and isolated *Aequorea* GFP proteins have been

-7-

used as markers for measuring gene expression, cell migration and localization, microtubule formation and assembly of functional ion channels (e.g., see Terry *et al.* (1995) Biochem. Biophys. Res. Commun. 217:21-27; Kain *et al.* (1995) Biotechniques 19:650-655). The *A. victoria* GFP, however, is not ideal for use in
5 analytical and diagnostic processes. Consequently GFP mutants have been selected with the hope of identifying mutants that have single excitation spectral peaks shifted to the red.

In fact a stated purpose in constructing such mutants has been to attempt to make the *A. victoria* GFP more like the GFP from *Renilla*, which has
10 thus far not been cloned, but which has properties that make it far more ideal for use as an analytical tool. For many practical applications, the spectrum of *Renilla* GFP would be preferable to that of the *Aequorea* GFP, because wavelength discrimination between different fluorophores and detection of resonance energy transfer are easier if the component spectra are tall and
15 narrow rather than low and broad [see, U.S. Patent No. 5,625,048]. Furthermore, the longer wavelength excitation peak (475 nm) of *Renilla* GFP is almost ideal for fluorescein filter sets and is resistant to photobleaching, but has lower amplitude than the shorter wavelength peak at 395 nm, which is more susceptible to photobleaching [Chalfie *et al.* (1994) Science 263:802-805].

20 There exists a phylogenetically diverse and largely unexplored repertoire of bioluminescent proteins that are a reservoir for future development. Many of these, such as nucleic acid encoding *Renilla* GFPs have not, despite concentrated efforts to do so.

For these reasons, it would be desirable to have a variety of new
25 luciferases and fluorescent proteins, particularly, *Renilla* GFP available rather than use mutants of *A. victoria* GFP. It has, not, however, been possible to clone the gene encoding any *Renilla* GFPs. It would also be desirable to have a variety of GFPs and luciferases available in order to optimize systems for particular applications and to improve upon existing methods. Therefore, it is
30 an object herein to provide isolated nucleic acids encoding heretofore unavailable luciferases and the protein encoded thereby. It is also an object herein to provide isolated nucleic acids encoding *Renilla* GFPs, GFPs from other

-8-

species, and luciferases from a variety of species, and the proteins encoded thereby. It is also an object herein to provide bioluminescence generating systems that include the luciferases, luciferins, and also include GFPs.

SUMMARY OF THE INVENTION

5 Isolated nucleic acids that encode fluorescent proteins and nucleic acids that encode luciferases are provided. Nucleic acid molecules encoding GFPs from *Renilla* and from *Ptilosarcus* are provided.

Nucleic acid molecules that encode the *Renilla mulleri* luciferase, a *Gaussia* species luciferase and a *Pleuromamma* species luciferase are provided. Nucleic
10 acid probes derived therefrom are also provided. Functionally equivalent nucleic acids, such as those that hybridize under conditions of high stringency to the disclosed molecules, are also contemplated.

Host cells, including bacterial, yeast and mammalian host cells, and plasmids for expression of the nucleic acids encoding each luciferase and GFP
15 and combinations of luciferases and GFPs are also provided in these hosts are also provided. The genes can be modified by substitution of codons optimized for expression in selected host cells or hosts, such as humans and other mammals, or can be mutagenized to alter the emission properties.

Luciferases

20 Recombinant host cells, including bacterial, yeast and mammalian cells, containing heterologous nucleic acid encoding a *Renilla mulleri* luciferase and the nucleic acid are provided. In preferred embodiments, the heterologous nucleic acid encodes the sequence of amino acids set forth in SEQ ID No. 18. In more preferred embodiments, the heterologous nucleic acid encodes the
25 sequence of nucleotides set forth in SEQ ID No. 17. Also provided are functionally equivalent nucleic acids, such as nucleic acid molecules that hybridize under moderate or high stringency to the sequence of nucleotides set forth in SEQ ID No. 17, particularly when using the probes provided herein.

Isolated nucleic acids that encode luciferases from *Gaussia* are provided
30 herein. In particular, nucleic acid fragments that encode *Gaussia princeps* luciferase, and nucleic acid probes derived therefrom are provided. In a particular embodiment, the luciferase is encoded by the sequence of nucleotides

-9-

set forth in SEQ ID No. 19. Also provided are nucleic acid molecules that hybridize under moderate or high stringency to the sequence of nucleotides set forth in SEQ ID No. 19, particularly when using probes provided herein. Probes derived from this nucleic acid that can be used in methods provided herein to

5 isolate luciferases from any *Gaussia* species are provided. In an exemplary embodiment, nucleic acid encoding *Gaussia princeps* luciferase is provided. This nucleic acid encodes the sequence of amino acids set forth in SEQ ID No. 20.

Nucleic acids that encode *Pleuromamma* luciferase are provided. In

10 particular, a nucleic acid molecule that encodes a *Pleuromamma* luciferase and the encoded luciferase are set forth in SEQ ID Nos. 28 and 29, respectively. Nucleic acid encoding a *Pleuromamma* luciferase has also been isolated.

Expression vectors that contain DNA encoding a *Renilla mulleri*, *Gaussia* or *Pleuromamma* luciferase linked in operational association with a promoter

15 element that allows for the constitutive or inducible expression of the luciferase are provided. In preferred embodiments, the vectors are capable of expressing the *Renilla mulleri* luciferase in a wide variety of host cells. Vectors for producing chimeric *Renilla mulleri* luciferase fusion proteins, preferably chimeric antibody-luciferase or acetylcholine esterase fusion proteins, containing a

20 promoter element and a multiple cloning site located upstream or downstream of DNA encoding *Renilla mulleri* luciferase are also provided.

Recombinant cells containing heterologous nucleic acid encoding a *Gaussia* luciferase are also provided. Purified *Gaussia* luciferases and compositions containing a *Gaussia* luciferase alone or in combination with at

25 other components of a bioluminescence-generating system, such as a *Renilla* green fluorescent protein, are provided. The *Gaussia* luciferase can be used, for example, to provide fluorescent illumination of novelty items or used in methods of detecting and visualizing neoplastic tissue and other tissues, detecting infectious agents using immunoassays, such homogenous immunoassays and in

30 vitro fluorescent-based screening assays using multi-well assay devices, or provided in kits for carrying out any of the above-described methods. In particular, the *Gaussia*

-10-

luciferase may be used in conjunction with suitable fluorescent proteins in assays provided herein.

Methods using the probes for the isolation and cloning of luciferase-encoding DNA in *Gaussia*, *Pleuromamma* and other species are also provided.

- 5 In preferred embodiments, the nucleic acid probes are degenerate probes of at least 14 nucleotides, preferably 16 to 30 nucleotides, that are based on amino acids set forth in SEQ ID No. 19 and or the sequence of nucleotides set forth in SEQ ID No. 29.

- Vectors containing DNA encoding a *Gaussia* luciferase or *Pleuromamma* luciferase are provided. In particular, expression vectors that contain DNA encoding the luciferase linked in operational association with a promoter element that allows for the constitutive or inducible expression of luciferase are provided. In preferred embodiments, the vectors are capable of expressing the luciferase in a wide variety of host cells. Vectors for producing chimeric luciferase fusion proteins (see, e.g., U.S. Patent No. 5,464,745, which describes the use of protein binding domains; see SEQ ID Nos. 21 and 22, which set forth the sequences of a cellulose binding domain-luciferase fusion protein; and which are depicted in FIGS. 1 and 2) containing a promoter element and a multiple cloning site located upstream or downstream from DNA encoding *Gaussia* or *Pleuromamma* luciferase are also provided. In a particular embodiment, DNA encoding the luciferase is linked to DNA encoding the N-terminal portion of the cellulose binding domain (CBD_{clos}; see, SEQ ID Nos. 21 and 22) in a PET vector (Novagen; see, U.S. Patent Nos. 5,719,044 and 5,738,984, 5,670,623 and 5,496,934 and the Novagen catalog; complete sequences of each PET vector are provided with purchase of the vector).

- Fusions of the nucleic acid, particularly DNA, encoding a *Gaussia* or *Pleuromamma* luciferase with DNA encoding a GFP or phycobiliprotein are also provided herein. Also provided are fusions of *Renilla* luciferase and a *Renilla* GFP. In these fusions the luciferase and GFP encoding DNA can be contiguous or separated by a spacer peptide. The fusions are used to produce fusion proteins, which by virtue of the interaction between the luciferase and GFP pair have a variety of unique analytical applications. The interaction is assessed by

-11-

the emission spectrum of the luciferase-GFP protein pair in the presence of a luciferin and appropriate binding factors.

Recombinant host cells containing heterologous nucleic acid encoding a *Gaussia* or *Pleuromamma* luciferase are provided. In certain embodiments, the recombina
5 tant cells that contain the heterologous DNA encoding the luciferase are produced by transfection with DNA encoding a luciferase or by introduction of RNA transcripts of DNA encoding the protein. The DNA may be introduced as a linear DNA fragment or may be included in an expression vector for stable or transient expression of the encoding DNA.

10 The cells that express functional luciferase may be used alone or in conjunction with a bioluminescence-generating system, in cell-based assays and screening methods, such as those described herein. Presently preferred host cells for expressing the luciferase are bacteria, yeasts, fungi, plant cells, insect cells and animal cells.

15 Purified *Gaussia*, *Pleuromamma* and *Renilla mulleri* luciferases are provided. These luciferases are preferably obtained by expression of the nucleic acid provided herein in prokaryotic or eukaryotic cells that contain the nucleic acid that encodes the luciferase protein; and isolation of the expressed protein.

20 Compositions containing the luciferases are provided. The compositions can take any of a number of forms, depending on the intended method of use therefor. In certain embodiments, for example, the compositions contain a *Gaussia* luciferase, *Gaussia* luciferase peptide or *Gaussia* luciferase fusion protein, formulated for use in luminescent novelty items, immunoassays, donors
25 in FET [fluorescent energy transfer] assays, FRET [fluorescent resonance energy transfer] assays, HTRF [homogeneous time-resolved fluorescence] assays or used in conjunction with multi-well assay devices containing integrated photodetectors, such as those described herein.

In more preferred embodiments, the bioluminescence-generating system
30 includes, in addition to the luciferase a *Renilla mulleri* or *Ptilosarcus* GFP. These compositions can be used in a variety of methods and systems, such as

-12-

included in conjunction with diagnostic systems for the *in vivo* detection of neoplastic tissues and other tissues, such as those methods described herein.

Combinations containing a first composition containing a luciferase and a second composition containing one or more additional components of a bioluminescence-
5 generating system for use with articles of manufacture to produce novelty items are provided. These novelty items are designed for entertainment, recreation and amusement, and include, but are not limited to: toys, particularly squirt guns, toy cigarettes, toy "Halloween" eggs, footbags and board/card games; finger paints and other paints, slimy play material; textiles, particularly
10 clothing, such as shirts, hats and sports gear suits, threads and yarns; bubbles in bubble making toys and other toys that produce bubbles; balloons; figurines; personal items, such as cosmetics, bath powders, body lotions, gels, powders and creams, nail polishes, make-up, toothpastes and other dentifrices, soaps, body paints, and bubble bath; items such as inks, paper; foods, such as
15 gelatins, icings and frostings; fish food containing luciferins and transgenic fish, particularly transgenic fish that express a luciferase; plant food containing a luciferin or luciferase, preferably a luciferin for use with transgenic plants that express luciferase; and beverages, such as beer, wine, champagne, soft drinks, and ice cubes and ice in other configurations; fountains, including liquid
20 "fireworks" and other such jets or sprays or aerosols of compositions that are solutions, mixtures, suspensions, powders, pastes, particles or other suitable form.

Any article of manufacture that can be combined with a bioluminescence-generating system as provided herein and thereby provide
25 entertainment, recreation and/or amusement, including use of the items for recreation or to attract attention, such as for advertising goods and/or services that are associated with a logo or trademark is contemplated herein. Such uses may be in addition to or in conjunction with or in place of the ordinary or normal use of such items. As a result of the combination, the items glow or produce,
30 such as in the case of squirt guns and fountains, a glowing fluid or spray of liquid or particles. The novelty in the novelty item derives from its bioluminescenc .

-13-

GFPS

Isolated nucleic acids that encode GFPs from *Renilla* are provided herein. Also provided are isolated and purified nucleic acids that encode a component of the bioluminescence generating system and a green fluorescent protein (GFP) of a member of the genus *Renilla*, and the proteins encoded thereby are provided. In particular, nucleic acid fragments that encode *Renilla* green fluorescent protein (GFPs) and the *Renilla mulleri* luciferase, and nucleic acid probes derived therefrom are provided.

Nucleic acid molecules encoding *Renilla* GFP are provided. In particular, nucleic acid molecules encoding a *Renilla* GFP that includes the coding portion of the sequence of nucleotides set forth in SEQ ID No. 15 or that hybridizes under moderate or high stringency to the sequence of nucleotides set forth in SEQ ID No. 15, particularly when using probes provided herein, are provided. Probes derived from this nucleic acid that can be used in methods provided herein to isolated GFPs from any *Renilla* species. In an exemplary embodiment, nucleic acid encoding *Renilla mulleri* GFP is provided. This nucleic acid encodes the sequence of amino acids set forth in SEQ ID No. 16.

Nucleic acid probes can be labeled, which if needed, for detection, containing at least about 14, preferably at least about 16, or, if desired, 20 or 30 or more, contiguous nucleotides of sequence of nucleotides encoding a *Renilla* GFP, particularly *Renilla mulleri*. In preferred embodiments, the nucleic acid probes for the *Renilla* GFP are selected from the sequence of nucleotides set forth in SEQ ID No. 15.

Methods using the probes for the isolation and cloning of GFP-encoding DNA in *Renilla* and other species are also provided. In preferred embodiments, the nucleic acid probes are degenerate probes based upon the conserved regions between the *Renilla* species of GFP as set forth in Figure 3. Such degenerate probes contain at least 14 nucleotides, preferably 16 to 30 nucleotides, that are based on amino acids 51 to 68, 82 to 98 and 198 to 208 set forth in SEQ ID No. 16, amino acid sequence set forth in SEQ ID No. 24, amino acids 9-20 set forth in SEQ ID No. 25 and amino acids 39-53 set forth in SEQ ID No. 27. In other preferred embodiments, the nucleic acid probes

-14-

encoding the above-described preferred amino acid regions are selected among the sequence of nucleotides encoding these regions as set forth in SEQ ID NO. 15. Alternatively, nucleic acids, particularly those set forth in SEQ ID No. 15 that encode the noted regions may be used as primers for PCR amplification of
5 libraries of a selected *Renilla* species, whereby DNA comprising that encodes a *Renilla* GFP is isolated.

Nucleic acids that encode a *Ptilosarcus* GFP are set forth in SEQ ID Nos. 30 and 31; the encoded GFP is set forth in SEQ ID No. 32. Also provided are nucleic acid molecules that hybridize under moderate or high stringency to the
10 sequence of nucleotides set forth in SEQ ID Nos. 28, 30 and 31.

Vectors containing DNA encoding a *Renilla* or *Ptilosarcus* GFP are provided. In particular, expression vectors that contain DNA encoding a *Renilla* or *Ptilosarcus* GFP linked in operational association with a promoter element that allows for the constitutive or inducible expression of *Renilla* or *Ptilosarcus*
15 GFP are provided. Native *Renilla* GFP has been expressed.

The vectors are capable of expressing the *Renilla* GFP in a wide variety of host cells. Vectors for producing chimeric *Renilla* GFP fusion proteins containing a promoter element and a multiple cloning site located upstream or downstream of DNA encoding *Renilla* GFP are also provided.

20 Recombinant cells containing heterologous nucleic acid encoding a *Ptilosarcus* GFP, *Renilla* GFP, *Renilla mulleri* luciferase, *Gaussia* luciferase, and *Pleuromamma* luciferase are also provided. Purified *Renilla mulleri* GFP, *Renilla reniformis* GFP peptides and compositions containing a *Renilla* GFPs and GFP peptides alone or in combination with at least one component of a
25 bioluminescence-generating system, such as a *Renilla mulleri* luciferase, are provided. The *Renilla* GFP and GFP peptide compositions can be used, for example, to provide fluorescent illumination of novelty items or used in methods of detecting and visualizing neoplastic tissue and other tissues, detecting infectious agents using immunoassays, such homogenous immunoassays and in
30 vitro fluorescent-based screening assays using multi-well assay devices, or provided in kits for carrying out any of the above-described methods. In particular, these proteins may be used in FP [fluorescence polarization] assays,

-15-

FET [fluorescent energy transfer] assays, FRET [fluorescent resonance energy transfer] assays and HTRF [homogeneous time-resolved fluorescence] assays and also in the BRET assays and sensors provided herein.

Non-radioactive energy transfer reactions, such as FET or FRET, FP and
5 HTRF assays, are homogeneous luminescence assays based on energy transfer are carried out between a donor luminescent label and an acceptor label [see, e.g., Cardullo *et al.* (1988) *Proc. Natl. Acad. Sci. U.S.A.* 85:8790-8794; Pearce *et al.* (1986) *Proc. Natl. Acad. Sci. U.S.A.* 83:8092-8096; U.S. Patent No. 4,777,128; U.S. Patent No. 5,162,508; U.S. Patent No. 4,927,923; U.S.
10 Patent No. 5,279,943; and International PCT Application No. WO 92/01225]. Non-radioactive energy transfer reactions using GFPs have been developed [see, International PCT application Nos. WO 98/02571 and WO 97/28261]. Non-radioactive energy transfer reactions using GFPs and luciferases, such as a luciferase and its cognate GFP (or multimers thereof), such as in a fusion
15 protein, are contemplated herein.

Nucleic acids that exhibit substantial sequence identity with the nucleic acids provided herein are also contemplated. These are nucleic acids that can be produced by substituting codons that encode conservative amino acids and also nucleic acids that exhibit at least about 80%, preferably 90 or 95%
20 sequence identity. Sequence identity refers to identity as determined using standard programs with default gap penalties and other defaults as provided by the manufacturer thereof.

The nucleic acids provide an opportunity to produce luciferases and GFPs, which have advantageous application in all areas in which
25 luciferase/luciferins and GFPs have application. The nucleic acids can be used to obtain and produce GFPs and GFPs from other, particularly *Renilla* species using the probes described herein that correspond to conserved regions (see, e.g., Figure 3). These GFPs have advantageous application in all areas in which GFPs and/or luciferase/luciferins have application. For example, The GFP's provide a
30 means to amplify the output signal of bioluminescence generating systems. *Renilla* GFP has a single excitation absorbance peak in blue light (and around 498 nm) and a predominantly single emission peak around 510 nm (with a small

-16-

shoulder near 540). This spectra provides a means for it to absorb blue light and efficiently convert it to green light. This results in an amplification of the output. When used in conjunction with a bioluminescence generating system that yields blue light, such as *Aequorea* or *Renilla* or *Vargula (Cypridina)*, the
5 output signal for any application, including diagnostic applications, is amplified. In addition, this green light can serve as an energy donor in fluorescence-based assays, such as fluorescence polarization assays, FET [fluorescent energy transfer] assays, FRET [fluorescent resonance energy transfer] assays and HTRF [homogeneous time-resolved fluorescence] assays. Particular assays, herein
10 referred to as BRET [bioluminescence resonance energy transfer assays in which energy is transferred from a bioluminescence reaction of a luciferase to a fluorescent protein], are provided.

Non-radioactive energy transfer reactions, such as FET or FRET, FP and HTRF assays, are homogeneous luminescence assays based on energy transfer
15 that are carried out between a donor luminescent label and an acceptor label [see, e.g., Cardullo *et al.* (1988) *Proc. Natl. Acad. Sci. U.S.A.* 85:8790-8794; Pearce *et al.* (1986) *Proc. Natl. Acad. Sci. U.S.A.* 83:8092-8096; U.S. Patent No. 4,777,128; U.S. Patent No. 5,162,508; U.S. Patent No. 4,927,923; U.S. Patent No. 5,279,943; and International PCT Application No. WO 92/01225].
20 Non-radioactive energy transfer reactions using GFPs have been developed [see, International PCT application Nos. WO 98/02571 and WO 97/28261].

Mutagenesis of the GFPs is contemplated herein, particularly
mutagenesis that results in modified GFPs that have red-shifted excitation and emission spectra. The resulting systems have higher output compared to the
25 unmutagenized forms. These GFPs may be selected by random mutagenesis and selection for GFPs with altered spectra or by selected mutagenesis of the chromophore region of the GFP.

Recombinant host cells containing heterologous nucleic acid encoding a
Renilla or *Ptilosarcus* GFP are also provided. In certain embodiments, the
30 recombinant cells that contain the heterologous DNA encoding the *Renilla* or *Ptilosarcus* GFP are produced by transfection with DNA encoding a *Renilla* or *Ptilosarcus* GFP or by introduction of RNA transcripts of DNA encoding a *Renilla*

-17-

or *Ptilosarcus* protein. The DNA may be introduced as a linear DNA fragment or may be included in an expression vector for stable or transient expression of the encoding DNA.

In certain embodiments, the cells contain DNA or RNA encoding a *Renilla*
5 *mulleri* GFP or a *Ptilosarcus* GFP (particularly from a species other than *P. gurneyi*) also express the recombinant *Renilla mulleri* GFP or *Ptilosarcus* polypeptide. It is preferred that the cells are selected to express functional GFPs that retain the ability to fluorescence and that are not toxic to the host cell. In some embodiments, cells may also include heterologous nucleic acid
10 encoding a component of a bioluminescence-generating system, preferably a photoprotein or luciferase. In preferred embodiments, the nucleic acid encoding the bioluminescence-generating system component is isolated from the species *Aequorea*, *Vargula*, *Pleuromamma*, *Ptilosarcus* or *Renilla*. In more preferred embodiments, the bioluminescence-generating system component is a *Renilla*
15 *mulleri* luciferase including the amino acid sequence set forth in SEQ ID No. 18 or the *Pleuromamma* luciferase set forth in SEQ ID No. 28, or the *Gaussia* luciferase set forth in SEQ ID No. 19.

The GFPs provided herein may be used in combination with any suitable bioluminescence generating system, but is preferably used in combination with
20 a *Renilla* or *Aequorea*, *Pleuromamma* or *Gaussia* luciferase.

Purified *Renilla* GFPs, particularly *Renilla mulleri* GFP, and purified *Renilla reniformis* GFP peptides are provided. Presently preferred *Renilla* GFP for use in the compositions herein is *Renilla mulleri* GFP including the sequence of amino acids set forth in SEQ ID No. 16. Presently preferred *Renilla reniformis* GFP
25 peptides are those containing the GFP peptides selected from the amino acid sequences set forth in SEQ ID Nos 19-23.

The *Renilla* GFP, GFP peptides and luciferase can be isolated from natural sources or isolated from a prokaryotic or eukaryotic cell transfected with nucleic acid that encodes the *Renilla* GFP and/or luciferase protein.

30 Fusions of the nucleic acid, particularly DNA, encoding *Renilla* or *Ptilosarcus* GFP with DNA encoding a luciferase are also provided herein.

-18-

The cells that express functional luciferase and/or GFP, which may be used alone or in conjunction with a bioluminescence-generating system, in cell-based assays and screening methods, such as those described herein.

Presently preferred host cells for expressing GFP and luciferase are
5 bacteria, yeasts, fungi, plant cells, insect cells and animal cells.

The luciferases and GFPs or cells that express them also may be used in methods of screening for bacterial contamination and methods of screening for metal contaminants. To screen for bacterial contamination, bacterial cells that express the luciferase and/or GFP are put in autoclaves or in other areas in
10 which testing is contemplated. After treatment or use of the area, the area is tested for the presence of glowing bacteria. Presence of such bacteria is indicative of a failure to eradicate other bacteria. Screening for heavy metals and other environmental contaminants can also be performed with cells that contain the nucleic acids provided herein, if expression is linked to a system
15 that is dependent upon the particular heavy metal or contaminant.

The systems and cells provided herein can be used for high throughout screening protocols, intracellular assays, medical diagnostic assays, environmental testing, such as tracing bacteria in water supplies, in conjunction with enzymes for detecting heavy metals, in spores for testing autoclaves in
20 hospital, foods and industrial autoclaves. Non-pathogenic bacteria containing the systems can be included in feed to animals to detect bacterial contamination in animal products and in meats.

Compositions containing a *Renilla* or *Ptilosarcus* GFP are provided. The compositions can take any of a number of forms, depending on the intended
25 method of use therefor. In certain embodiments, for example, the compositions contain a *Renilla* GFP or GFP peptide, preferably *Renilla mulleri* GFP or *Renilla reniformis* GFP peptide, formulated for use in luminescent novelty items, immunoassays, FET [fluorescent energy transfer] assays, FRET [fluorescent resonance energy transfer] assays, HTRF [homogeneous time-resolved
30 fluorescence] assays or used in conjunction with multi-well assay devices containing integrated photodetectors, such as those described herein. In other instances, the GFPs are used in beverages, foods or cosmetics.

-19-

Compositions that contain a *Renilla mulleri* GFP or GFP peptide and at least one component of a bioluminescence-generating system, preferably a luciferase, luciferin or a luciferase and a luciferin, are provided. In preferred embodiments, the luciferase/luciferin bioluminescence-generating system is
5 selected from those isolated from: an insect system, a coelenterate system, a ctenophore system, a bacterial system, a mollusk system, a crustacea system, a fish system, an annelid system, and an earthworm system. Bioluminescence-generating systems include those isolated from *Renilla*, *Aequorea*, and *Vargula*, *Gaussia* and *Pleuromamma*.

10 Combinations containing a first composition containing a *Renilla mulleri* GFP or *Ptilosarcus* GFP or mixtures thereof and a second composition containing a bioluminescence-generating system for use with inanimate articles of manufacture to produce novelty items are provided. These novelty items,
15 which are articles of manufacture, are designed for entertainment, recreation and amusement, and include, but are not limited to: toys, particularly squirt guns, toy cigarettes, toy "Halloween" eggs, footbags and board/card games; finger paints and other paints, slimy play material; textiles, particularly clothing, such as shirts, hats and sports gear suits, threads and yarns; bubbles in bubble making toys and other toys that produce bubbles; balloons; figurines; personal
20 items, such as bath powders, body lotions, gels, powders and creams, nail polishes, cosmetics including make-up, toothpastes and other dentifrices, soaps, cosmetics, body paints, and bubble bath, bubbles made from non-detergent sources, particularly proteins such as albumin and other non-toxic proteins; in fishing lures, particularly crosslinked polyacrylamide containing a fluorescent
25 protein and/or components of a bioluminescence generating system, which glow upon contact with water; items such as inks, paper; foods, such as gelatins, icings and frostings; fish food containing luciferins and transgenic fish, particularly transgenic fish that express a luciferase; plant food containing a luciferin or luciferase, preferably a luciferin for use with transgenic plants that
30 express luciferase; and beverages, such as beer, wine, champagne, soft drinks, and ice cubes and ice in other configurations; fountains, including liquid "fireworks" and other such jets or sprays or aerosols of compositions that are

-20-

solutions, mixtures, suspensions, powders, pastes, particles or other suitable form.

Any article of manufacture that can be combined with a bioluminescence-generating system as provided herein and thereby provide
5 entertainment, recreation and/or amusement, including use of the items for recreation or to attract attention, such as for advertising goods and/or services that are associated with a logo or trademark is contemplated herein. Such uses may be in addition to or in conjunction with or in place of the ordinary or normal use of such items. As a result of the combination, the items glow or produce,
10 such as in the case of squirt guns and fountains, a glowing fluid or spray of liquid or particles.

Methods for diagnosis and visualization of tissues *in vivo* or *in situ* using compositions containing a *Renilla mulleri* GFP and/or a *Renilla mulleri* luciferase or others of the luciferases and/or GFPs provided herein are provided. For
15 example, the *Renilla mulleri* GFP protein can be used in conjunction with diagnostic systems that rely on bioluminescence for visualizing tissues *in situ*. The systems are particularly useful for visualizing and detecting neoplastic tissue and specialty tissue, such as during non-invasive and invasive procedures. The systems include compositions containing conjugates that
20 include a tissue specific, particularly a tumor-specific, targeting agent linked to a targeted agent, a *Renilla mulleri* GFP, a luciferase or luciferin. The systems also include a second composition that contains the remaining components of a bioluminescence generating reaction and/or the *Renilla mulleri* GFP. In some embodiments, all components, except for activators, which are provided *in situ*
25 or are present in the body or tissue, are included in a single composition.

Methods for diagnosis and visualization of tissues *in vivo* or *in situ* using compositions containing a *Gaussia* luciferase are provided. For example, the *Gaussia* luciferase or *Gaussia* luciferase peptide can be used in conjunction with diagnostic systems that rely on bioluminescence for visualizing tissues *in situ*.
30 The systems are particularly useful for visualizing and detecting neoplastic tissue and specialty tissue, such as during non-invasive and invasive procedures. The systems include compositions containing conjugates that

-21-

include a tissue specific, particularly a tumor-specific, targeting agent linked to a targeted agent, a *Gaussia* luciferase, a GFP or luciferin. The systems also include a second composition that contains the remaining components of a bioluminescence generating reaction and/or the *Gaussia* luciferase. In some
5 embodiments, all components, except for activators, which are provided *in situ* or are present in the body or tissue, are included in a single composition.

 In particular, the diagnostic systems include two compositions. A first composition that contains conjugates that, in preferred embodiments, include antibodies directed against tumor antigens conjugated to a component of the
10 bioluminescence generating reaction, a luciferase or luciferin, preferably a luciferase are provided. In certain embodiments, conjugates containing tumor-specific targeting agents are linked to luciferases or luciferins. In other embodiments, tumor-specific targeting agents are linked to microcarriers that are coupled with, preferably more than one of the bioluminescence generating
15 components, preferably more than one luciferase molecule.

 The second composition contains the remaining components of a bioluminescence generating system, typically the luciferin or luciferase substrate. In some embodiments, these components, particularly the luciferin are linked to a protein, such as a serum albumin, or other protein carrier. The
20 carrier and time release formulations, permit systemically administered components to travel to the targeted tissue without interaction with blood cell components, such as hemoglobin that deactivates the luciferin or luciferase..

 Methods for diagnosing diseases, particularly infectious diseases, using chip methodology (see, e.g., copending U.S. application Serial No. 08/990,103)
25 a luciferase/luciferin bioluminescence-generating system and a *Renilla mulleri* or *Ptilosarcus* GFP are provided. In particular, the chip includes an integrated photodetector that detects the photons emitted by the bioluminescence-generating system, particularly using luciferase encoded by the nucleic acids provided herein and/or *Renilla mulleri* or *Ptilosarcus* GFP.

30 In one embodiment, the chip is made using an integrated circuit with an array, such as an X-Y array, of photodetectors. The surface of circuit is treated to render it inert to conditions of the diagnostic assays for which the chip is

-22-

- intended, and is adapted, such as by derivatization for linking molecules, such as antibodies. A selected antibody or panel of antibodies, such as an antibody specific for a bacterial antigen, is affixed to the surface of the chip above each photodetector. After contacting the chip with a test sample, the chip is
- 5 contacted with a second antibody linked to a *Renilla* or *Pleuromamm* GFP, a chimeric antibody-*Renilla* GFP fusion protein or an antibody linked to a component of a bioluminescence generating system, such as a luciferase or luciferin, that are specific for the antigen. The remaining components of the bioluminescence generating reaction are added, and, if any of the antibodies
- 10 linked to a component of a bioluminescence generating system are present on the chip, light will be generated and detected by the adjacent photodetector. The photodetector is operatively linked to a computer, which is programmed with information identifying the linked antibodies, records the event, and thereby identifies antigens present in the test sample.
- 15 Methods for generating chimeric GFP fusion proteins are provided. The methods include linking DNA encoding a gene of interest, or portion thereof, to DNA encoding a GFP coding region in the same translational reading frame. The encoded-protein of interest may be linked in-frame to the amino- or carboxyl-terminus of the GFP. The DNA encoding the chimeric protein is then linked in
- 20 operable association with a promoter element of a suitable expression vector. Alternatively, the promoter element can be obtained directly from the targeted gene of interest and the promoter-containing fragment linked upstream of the GFP coding sequence to produce chimeric GFP proteins.
- Methods for identifying compounds using recombinant cells that express
- 25 heterologous DNA encoding a GFP under the control of a promoter element of a gene of interest are provided. The recombinant cells can be used to identify novel compounds or ligands that modulate the level of transcription from the promoter of interest by measuring GFP-mediated fluorescence. Recombinant cells expressing the chimeric *Renilla* or *Ptilosarcus* GFPs may also be used for
- 30 monitoring gene expression or protein trafficking, or determining the cellular localization of the target protein by identifying localized regions of GFP-mediated fluorescence within the recombinant cell.

-23-

Other assays using the GFPs and/or luciferases are contemplated herein. Any assay or diagnostic method known used by those of skill in the art that employ *Aequora* GFPs and/or other luciferases are contemplated herein.

Kits containing the GFPs for use in the methods, including those
5 described herein, are provided. In one embodiment, the kits containing an article of manufacture and appropriate reagents for generating bioluminescence are provided. The kits containing such soap compositions, with preferably a moderate Ph [between 5 and 8] and bioluminescence generating reagents, including luciferase and luciferin and the GFP are provided herein. These kits,
10 for example, can be used with a bubble-blowing or producing toy. These kits can also include a reloading or charging cartridge or can be used in connection with a food.

In another embodiment, the kits are used for detecting and visualizing neoplastic tissue and other tissues and include a first composition that contains
15 the GFP and at least one component of a bioluminescence generating system, and a second that contains the activating composition, which contains the remaining components of the bioluminescence generating system and any necessary activating agents.

Thus, these kits will typically include two compositions, a first
20 composition containing the GFP formulated for systemic administration (or in some embodiments local or topical application), and a second composition containing the components or remaining components of a bioluminescence generating system, formulated for systemic, topical or local administration depending upon the application. Instructions for administration will be included.

25 In other embodiments, the kits are used for detecting and identifying diseases, particularly infectious diseases, using multi-well assay devices and include a multi-well assay device containing a plurality of wells, each having an integrated photodetector, to which an antibody or panel of antibodies specific for one or more infectious agents are attached, and composition containing a
30 secondary antibody, such as an antibody specific for the infectious agent that is linked to a *Renilla mulleri* or *Ptilosarcus* GFP protein, a chimeric antibody-*Renilla mulleri* (or *Ptilosarcus*) GFP fusion protein or F(Ab)₂ antibody fragment-*Renilla*

-24-

mulleri GFP fusion protein. A second composition containing a bioluminescence generating system that emits a wavelength of light within the excitation range of the *Renilla mulleri* GFP, such as species of *Renilla* or *Aequorea*, for exciting the *Renilla mulleri*, which produces green light that is detected by the
5 photodetector of the device to indicate the presence of the agent.

As noted above, fusions of nucleic acid encoding the luciferases and or GFPs provided herein with other luciferases and GFPs are provided. Of particular interest are fusions that encode pairs of luciferases and GFPs, such as a *Renilla* luciferase and a *Renilla* GFP (or a homodimer or other multiple of a
10 *Renilla* GFP). The luciferase and GFP bind and in the presence of a luciferin will produced fluorescence that is red shifted compared to the luciferase in the absence of the GFP. This fusion or fusions in which the GFP and luciferase are linked via a target, such as a peptide, can be used as a tool to assess anything that interacts with the linker.

15 Muteins of the GFPs and luciferases are provided. Of particular interest are muteins, such as temperature sensitive muteins, of the GFP and luciferases that alter their interaction, such as mutations in the *Renilla* luciferase and *Renilla* GFP that alters their interaction at a critical temperature.

Antibodies, polyclonal and monoclonal antibodies that specifically bind to
20 any of the proteins encoded by the nucleic acids provided herein are also provided. These antibodies, monoclonal or polyclonal, can be prepared employing standard techniques, known to those of skill in the art. In particular, immunoglobulins or antibodies obtained from the serum of an animal immunized with a substantially pure preparation of a luciferase or GFP provided herein or an
25 or epitope-containing fragment thereof are provided. Monoclonal antibodies are also provided. The immunoglobulins that are produced have, among other properties, the ability to specifically and preferentially bind to and/or cause the immunoprecipitation of a GFP or luciferase, particularly a *Renilla* or *Ptsilocarpus* GFP or a *Pleuromamma*, *Gaussia* or *Renilla mulleri* luciferase, that may be
30 present in a biological sample or a solution derived from such a biological sample.

-25-

DESCRIPTION OF THE FIGURES

FIGURE 1 depicts the components of the commercially available PET-34 vector (EK is enterokinase).

FIGURE 2 shows a portion of the vector with the inserted *Gaussia*-
5 encoding luciferase.

FIGURE 3 displays an alignment of the deduced amino acid sequence of *Renilla mulleri* green fluorescent protein and the amino acid sequence of isolated *Renilla reniformis* GFP peptides obtained by proteolytic digestion of purified *Renilla reniformis* GFP. Positions in the amino acid sequence of direct identity
10 are marked by the solid vertical lines (|) between the two *Renilla* species.

FIGURE 4 shows the fluorescence emission and excitation spectrum for the *Renilla mulleri* GFP, with a peak emission at 506 nm..

FIGURE 5 shows the fluorescence emission and excitation spectrum for the *Ptilosarcus* GFP, with a peak emission at 508.

FIGURE 6 shows a photoemission as a function of salt and Ph for
15 *Pleuromamma* luciferase.

FIGURE 7 depicts the components of a *Renilla mulleri* luciferase-GFP fusion construct in pET-34; rbs: ribosome binding sequence; CDS: coding domain sequence; CBD: the cellulose binding domain; thrombin: thrombin
20 cleavage site; EK: enterokinase cleavage site; S Tag: the RNase-S-peptide tag; and LIC: ligation independent cloning site.

FIGURE 8 shows a photoemission as a function of salt and pH for a *Gaussia* luciferase.

FIGURE 9 shows a photoemission spectrum for a *Gaussia* luciferase.

FIGURE 10 shows a photoemission spectrum for a *Pleuromamma*
25 luciferase.

FIGURE 11 illustrates the underlying principle of Bioluminescent Resonance Energy Transfer (BRET) and its use as sensor: A) in isolation, a luciferase, preferably an anthozoan luciferase, emits blue light from the
30 coelenterazine-derived chromophore; B) in isolation, a GFP, preferably an anthozoan GFP that binds to the luciferase, that is excited with blue-green light emits green light from its integral peptide based flurophore; C) when the

luciferase and GFP associate as a complex *in vivo* or *in vitro*, the luciferase non-radiatively transfers its reaction energy to the GFP flurophore, which then emits the green light; D) any molecular interaction that disrupts the luciferase-GFP complex can be quantitatively monitored by observing the spectral shift from green to blue light.

DETAILED DESCRIPTION OF THE INVENTION

TABLE OF CONTENTS

	A.	DEFINITIONS
	B.	BIOLUMINESCENT GENERATING SYSTEMS
10	1.	Exemplary bioluminescence generating systems
	a.	General description
		(1) Luciferases
		(2) Luciferins
		(3) Activators
15		(4) Reactions
	b.	Ctenophore systems
		(1) The aequorin system
		(a) Aequorin and related photoproteins
		(b) Luciferin
20		(2) The <i>Renilla</i> system
	c.	Crustacean, particularly <i>Cypridina</i> systems
		(1) <i>Vargula</i> luciferase
		(a) Purification from <i>Cypridina</i>
		(b) Preparation by Recombinant Methods
25		(2) <i>Vargula</i> luciferin
		(3) Reaction
	d.	Insect bioluminescent systems including fireflies, click beetles, and other insect system
30		(1) Luciferase
		(2) Luciferin
		(3) Reaction
	e.	Bacterial systems
		(1) Luciferases
		(2) Luciferins

-27-

- (3) Reactions
 - f. Other systems
 - (1) Dinoflagellate bioluminescence generating systems
 - (2) Systems from molluscs, such as *Latia* and *Pholas*
 - 5 (3) Earthworms and other annelids
 - (4) Glow worms
 - (5) Marine polychaete worm systems
 - (6) South American railway beetle
 - (7) Fish
 - 10 g. Fluorescent proteins
 - (1) Green and blue fluorescent proteins
 - (2) Phycobiliproteins
- C. ISOLATION AND IDENTIFICATION OF NUCLEIC ACIDS ENCODING LUCIFERASES
 - 15 ISOLATION AND IDENTIFICATION OF NUCLEIC ACID ENCODING *Gaussia* LUCIFERASE
 - 1. Isolation of specimens of the genus *Gaussia*
 - 2. Preparation of *Gaussia* cDNA expression libraries
 - 20 3. Isolation and identification of DNA encoding *Gaussia* luciferase
 - ISOLATION AND IDENTIFICATION OF NUCLEIC ACID ENCODING *Renilla* PROTEINS
 - 1. Isolation of specimens of the genus *Renilla*
 - 2. Preparation of *Renilla* cDNA expression libraries
 - 25 a. RNA isolation and cDNA synthesis
 - b. Construction of cDNA expression libraries
 - 30 3. Isolation and identification of DNA encoding *Renilla* GFP
 - 4. Isolation and identification of DNA encoding *Renilla* luciferase
- D. NUCLEIC ACID PROBES AND METHODS FOR ISOLATING AND CLONING LUCIFERASE- and GFP-ENCODING NUCLEIC ACIDS FROM OTHER SPECIES
 - Gaussia*
 - 35 NUCLEIC ACID PROBES AND METHODS FOR ISOLATING AND CLONING OF GFP-ENCODING NUCLEIC ACIDS FROM OTHER SPECIES OF *Renilla*
 - Other species
- E. RECOMBINANT EXPRESSION OF PROTEINS

-28-

Gaussia

1. DNA encoding *Gaussia* proteins
2. DNA constructs for recombinant production of *Gaussia* proteins
3. Host organisms for recombinant production of *Gaussia* proteins
4. Methods for recombinant production of *Gaussia* proteins

Renilla

1. DNA encoding *Renilla* proteins
2. DNA constructs for recombinant production of *Renilla* proteins
3. Host organisms for recombinant production of *Renilla* proteins
4. Methods for recombinant production of *Renilla* proteins

F. RECOMBINANT CELLS EXPRESSING HETEROLOGOUS NUCLEIC ACID ENCODING A LUCIFERASES AND GFPs

*Gaussia*15 RECOMBINANT CELLS EXPRESSING HETEROLOGOUS NUCLEIC ACID ENCODING A *Renilla* GREEN FLUORESCENT PROTEIN AND/OR LUCIFERASE

G. Luciferases

H. *Renilla* and *Ptilosarcus* GFPs

I. COMPOSITIONS

1. *Gaussia* luciferase compositions
2. *Renilla* luciferase compositions
3. *Renilla* GFP compositions
4. Conjugates
 - a. Linkers
 - b. Targeting Agents
 - Anti-tumor Antigen Antibodies
 - Preparation of the conjugates
5. Formulation of the compositions for use in the
 - a. The first composition: formulation of the conjugates diagnostic systems
 - b. The second composition
 - c. Practice of the reactions in combination with targeting agents

J. COMBINATIONS

K. METHODS OF USE

1. Methods for diagnosis of neoplasms and other tissues
2. Methods of diagnosing diseases

-29-

3. Methods for generating chimeric *Renilla* or *Ptilosarcus* GFP, *Renilla mulleri* luciferase, *Pleuromamma* luciferase and *Gaussia* luciferase fusion proteins
4. Cell-based assays for identifying compounds
- 5 L. KITS
 1. Dispensing and Packaging Apparatus for Combination with the GFP and Bioluminescent System Components
 2. Capsules, pellets, liposomes, endosomes, vacuoles, micronized particles
 - a. Encapsulating vehicles in general
 - 10 b. Encapsulating vehicles -liposomes
 - c. Encapsulating vehicles -gelatin and polymeric vehicles
 - d. Endosomes and vacuoles
 - e. Micronized particles
 3. Immobilized systems
 - 15 a. Matrix materials
 - b. Immobilization and activation

M. Bioluminescence Resonance Energy Transfer (BRET) System

N. EXAMPLES

A. DEFINITIONS

20 Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. All patents and publications of referred to herein are incorporated by reference in their entirety.

25 As used herein, chemiluminescence refers to a chemical reaction in which energy is specifically channeled to a molecule causing it to become electronically excited and subsequently to release a photon thereby emitting visible light. Temperature does not contribute to this channeled energy. Thus, chemiluminescence involves the direct conversion of chemical energy to light energy.

30 As used herein, luminescence refers to the detectable EM radiation, generally, UV, IR or visible EM radiation that is produced when the excited product of an exergic chemical process reverts to its ground state with the emission of light. Chemiluminescence is luminescence that results from a chemical reaction. Bioluminescence is chemiluminescence that results from a

-30-

chemical reaction using biological molecules [or synthetic versions or analogs thereof] as substrates and/or enzymes.

As used herein, bioluminescence, which is a type of chemiluminescence, refers to the emission of light by biological molecules, particularly proteins. The
5 essential condition for bioluminescence is molecular oxygen, either bound or free in the presence of an oxygenase, a luciferase, which acts on a substrate, a luciferin. Bioluminescence is generated by an enzyme or other protein [luciferase] that is an oxygenase that acts on a substrate luciferin [a bioluminescence substrate] in the presence of molecular oxygen and transforms
10 the substrate to an excited state, which upon return to a lower energy level releases the energy in the form of light.

As used herein, the substrates and enzymes for producing bioluminescence are generically referred to as luciferin and luciferase, respectively. When reference is made to a particular species thereof, for clarity,
15 each generic term is used with the name of the organism from which it derives, for example, bacterial luciferin or firefly luciferase.

As used herein, luciferase refers to oxygenases that catalyze a light emitting reaction. For instance, bacterial luciferases catalyze the oxidation of flavin mononucleotide [FMN] and aliphatic aldehydes, which reaction produces
20 light. Another class of luciferases, found among marine arthropods, catalyzes the oxidation of *Cypridina* [*Vargula*] luciferin, and another class of luciferases catalyzes the oxidation of *Coleoptera* luciferin.

Thus, luciferase refers to an enzyme or photoprotein that catalyzes a bioluminescent reaction [a reaction that produces bioluminescence]. The
25 luciferases, such as firefly and *Gaussia* and *Renilla* luciferases, that are enzymes which act catalytically and are unchanged during the bioluminescence generating reaction. The luciferase photoproteins, such as the aequorin photoprotein to which luciferin is non-covalently bound, are changed, such as by release of the luciferin, during bioluminescence generating reaction. The
30 luciferase is a protein that occurs naturally in an organism or a variant or mutant thereof, such as a variant produced by mutagenesis that has one or more properties, such as thermal stability, that differ from the naturally-occurring

-31-

protein. Luciferases and modified mutant or variant forms thereof are well known. For purposes herein, reference to luciferase refers to either the photoproteins or luciferases.

Thus, reference, for example, to "*Gaussia* luciferase" means an enzyme
 5 isolated from member of the genus *Gaussia* or an equivalent molecule obtained from any other source, such as from another related copepod, or that has been prepared synthetically. It is intended to encompass *Gaussia* luciferases with conservative amino acid substitutions that do not substantially alter activity. Suitable conservative substitutions of amino acids
 10 are known to those of skill in this art and may be made generally without altering the biological activity of the resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, e.g., Watson et al. *Molecular Biology of the Gene*, 4th Edition, 1987, The
 15 Bejacmin/Cummings Pub. co., p.224).

"*Renilla* GFP" refers to GFPs from the genus *Renilla* and to mutants or variants thereof. It is intended to encompass *Renilla* GFPs with conservative amino acid substitutions that do not substantially alter activity.

Such substitutions are preferably made in accordance with those set
 20 forth in TABLE 1 as follows:

TABLE 1

	Original residue	Conservative substitution
	Ala (A)	Gly; Ser
	Arg (R)	Lys
25	Asn (N)	Gln; His
	Cys (C)	Ser
	Gln (Q)	Asn
	Glu (E)	Asp
	Gly (G)	Ala; Pro
30	His (H)	Asn; Gln
	Ile (I)	Leu; Val
	Leu (L)	Ile; Val
	Lys (K)	Arg; Gln; Glu
	Met (M)	Leu; Tyr; Ile
35	Phe (F)	Met; Leu; Tyr
	Ser (S)	Thr
	Thr (T)	Ser

-32-

Original residue

Trp (W)

Tyr (Y)

Val (V)

Conservative substitution

Tyr

Trp; Phe

Ile; Leu

Other substitutions are also permissible and may be determined empirically or in
 5 accord with known conservative substitutions.

The luciferases and luciferin and activators thereof are referred to as
 bioluminescence generating reagents or components. Typically, a subset of
 these reagents will be provided or combined with an article of manufacture.
 Bioluminescence will be produced upon contacting the combination with the
 10 remaining reagents. Thus, as used herein, the component luciferases, luciferins,
 and other factors, such as O_2 , Mg^{2+} , Ca^{2+} are also referred to as
 bioluminescence generating reagents [or agents or components].

As used herein, bioluminescence substrate refers to the compound that
 is oxidized in the presence of a luciferase, and any necessary activators, and
 15 generates light. These substrates are referred to as luciferins herein, are
 substrates that undergo oxidation in a bioluminescence reaction. These
 bioluminescence substrates include any luciferin or analog thereof or any
 synthetic compound with which a luciferase interacts to generate light.
 Preferred substrates are those that are oxidized in the presence of a luciferase
 20 or protein in a light-generating reaction. Bioluminescence substrates, thus,
 include those compounds that those of skill in the art recognize as luciferins.
 Luciferins, for example, include firefly luciferin, *Cypridina* [also known as
Vargula] luciferin [coelenterazine], bacterial luciferin, as well as synthetic
 analogs of these substrates or other compounds that are oxidized in the
 25 presence of a luciferase in a reaction the produces bioluminescence.

As used herein, capable of conversion into a bioluminescence substrate
 means susceptible to chemical reaction, such as oxidation or reduction, that
 yields a bioluminescence substrate. For example, the luminescence producing
 reaction of bioluminescent bacteria involves the reduction of a flavin
 30 mononucleotide group (FMN) to reduced flavin mononucleotide ($FMNH_2$) by a
 flavin reductase enzyme. The reduced flavin mononucleotide [substrate] then
 reacts with oxygen [an activator] and bacterial luciferase to form an

-33-

intermediate peroxy flavin that undergoes further reaction, in the presence of a long-chain aldehyde, to generate light. With respect to this reaction, the reduced flavin and the long chain aldehyde are substrates.

As used herein, a bioluminescence generating system refers to the set of reagents required to conduct a bioluminescent reaction. Thus, the specific luciferase, luciferin and other substrates, solvents and other reagents that may be required to complete a bioluminescent reaction form a bioluminescence system. Thus a bioluminescence generating system refers to any set of reagents that, under appropriate reaction conditions, yield bioluminescence.

Appropriate reaction conditions refers to the conditions necessary for a bioluminescence reaction to occur, such as pH, salt concentrations and temperature. In general, bioluminescence systems include a bioluminescence substrate, luciferin, a luciferase, which includes enzymes luciferases and photoproteins, and one or more activators. A specific bioluminescence system may be identified by reference to the specific organism from which the luciferase derives; for example, the *Vargula* [also called *Cypridina*] bioluminescence system (or *Vargula* system) includes a *Vargula* luciferase, such as a luciferase isolated from the ostracod, *Vargula* or produced using recombinant means or modifications of these luciferases. This system would also include the particular activators necessary to complete the bioluminescence reaction, such as oxygen and a substrate with which the luciferase reacts in the presence of the oxygen to produce light.

The luciferases provided herein may be incorporated into bioluminescence generating systems and used, as appropriate, with the GFPs provided herein or with other GFPs. Similarly, the GFPs provided herein may be used with known bioluminescence generating systems.

As used herein, the amino acids, which occur in the various amino acid sequences appearing herein, are identified according to their well-known, three-letter or one-letter abbreviations. The nucleotides, which occur in the various DNA fragments, are designated with the standard single-letter designations used routinely in the art.

-34-

As used herein, a fluorescent protein refers to a protein that possesses the ability to fluoresce (i.e., to absorb energy at one wavelength and emit it at another wavelength). These proteins can be used as a fluorescent label or marker and in any applications in which such labels would be used, such as
5 immunoassays, CRET, FRET, and FET assays, and in the assays designated herein as BRET assays. For example, a green fluorescent protein refers to a polypeptide that has a peak in the emission spectrum at about 510 nm.

As used herein, the term BRET (Bioluminescence Resonance Energy Transfer) refers to non-radiative luciferase-to-FP energy transfer. It differs from
10 (Fluorescence Resonance Energy Transfer), which historically has been used for energy transfer between chemical fluors, but more recently has been applied to energy transfer between *Aequorea* GFP spectral variants.

As used herein, a BRET system refers the combination of a FP and luciferase for resonance energy transfer and BRET refers to any method
15 in which the luciferase is used to generate the light upon reaction with a luciferin which is then non-radiatively transferred to a FP. The energy is transferred to a FP, particularly a GFP, which focuses and shifts the energy and emits it at a different wavelength. In preferred embodiments, the BRET system includes a bioluminescence generating system and a GFP from the same source
20 as the luciferase in the system. A preferred pair is a *Renilla* luciferase and a *Renilla* GFP, which specifically interact. Alterations in the binding will be reflected in changes in the emission spectra of light produced by the luciferase. As a result the pair can function as a sensor of external events.

As used herein, a biosensor (or sensor) refers to a BRET system for use
25 to detect alterations in the environment *in vitro* or *in vivo* in which the BRET system is used.

As used herein, "not strictly catalytically" means that the photoprotein acts as a catalyst to promote the oxidation of the substrate, but it is changed in the reaction, since the bound substrate is oxidized and bound molecular oxygen
30 is used in the reaction. Such photoproteins are regenerated by addition of the substrate and molecular oxygen under appropriate conditions known to those of skill in this art.

-35-

As used herein, a nucleic acid probe is single-stranded DNA or RNA that has a sequence of nucleotides that includes at least 14 contiguous bases, preferably at least 16 contiguous bases, typically about 30, that are the same as (or the complement of) any 14 or more contiguous bases set forth in any of
5 SEQ ID No., particularly SEQ ID Nos 15, 19, 21, 28, 30, 31 and also nucleic acid that encodes any of the peptides in SEQ ID Nos. 23-27. Among the preferred regions from which to construct probes include 5' and/or 3' coding sequences, sequences predicted to encode regions that are conserved among *Renilla* species. Probes from regions conserved among *Renilla* species GFPs
10 are for isolating GFP-encoding nucleic acid from *Renilla* libraries.

In preferred embodiments, the nucleic acid probes are degenerate probes of at least 14 nucleotides, preferably 16 to 30 nucleotides, that are based on amino acids 51 to 68, 82 to 98 and 198 to 208 set forth in SEQ ID No. 16, amino acid sequence set forth in SEQ ID No. 24, amino acids 9-20 set forth in
15 SEQ ID No. 25 and amino acids 39-53 set forth in SEQ ID No. 27. In other preferred embodiments, the nucleic acid probes encoding the above-described preferred amino acid regions are selected among the sequence of nucleotides encoding these regions set forth in SEQ ID NO. 15.

In preferred embodiments, the nucleic acid probes are degenerate probes
20 of at least 14 nucleotides, preferably 16 to 30 nucleotides, that are based on amino acids set forth in SEQ ID No. 20. In other preferred embodiments, the nucleic acid probes encoding the above-described preferred amino acid regions are selected among the sequence of nucleotides encoding these regions set forth in SEQ ID NO. 19.

25 As used herein, vector (or plasmid) refers to discrete elements that are used to introduce heterologous DNA into cells for either expression or replication thereof. Selection and use of such vehicles are well within the skill of the artisan. An expression vector includes vectors capable of expressing DNAs that are operatively linked with regulatory sequences, such as promoter
30 regions, that are capable of effecting expression of such DNA fragments. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into

-36-

an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome. Presently
5 preferred plasmids for expression of *Gaussia* luciferase, *Renilla* GFP and luciferase are those that are expressed in bacteria and yeast, such as those described herein.

As used herein, a promoter region or promoter element refers to a segment of DNA or RNA that controls transcription of the DNA or RNA to which
10 it is operatively linked. The promoter region includes specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of RNA polymerase. These
15 sequences may be *cis* acting or may be responsive to *trans* acting factors. Promoters, depending upon the nature of the regulation, may be constitutive or regulated. Exemplary promoters contemplated for use in prokaryotes include the bacteriophage T7 and T3 promoters, and the like.

As used herein, operatively linked or operationally associated refers to
20 the functional relationship of DNA with regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of DNA to a promoter refers to the physical and functional relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the
25 promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA. In order to optimize expression and/or *in vitro* transcription, it may be necessary to remove, add or alter 5' untranslated portions of the clones to eliminate extra, potential inappropriate alternative translation initiation (i.e., start) codons or other sequences that may interfere
30 with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites (see, e.g., Kozak (1991) J. Biol. Chem. 266:19867-19870) can be inserted immediately 5' of the start codon

-37-

and may enhance expression. The desirability of (or need for) such modification may be empirically determined.

As used herein, to target a targeted agent, such as a luciferase, means to direct it to a cell that expresses a selected receptor or other cell surface
5 protein by linking the agent to a such agent. Upon binding to or interaction with the receptor or cell surface protein the targeted agent, can be reacted with an appropriate substrate and activating agents, whereby bioluminescent light is produced and the tumorous tissue or cells distinguished from non-tumorous tissue.

10 As used herein, an effective amount of a compound for treating a particular disease is an amount that is sufficient to ameliorate, or in some manner reduce the symptoms associated with the disease. Such amount may be administered as a single dosage or may be administered according to a regimen, whereby it is effective. The amount may cure the disease but,
15 typically, is administered in order to ameliorate the symptoms of the disease. Repeated administration may be required to achieve the desired amelioration of symptoms.

As used herein, an effective amount of a conjugate for diagnosing a disease is an amount that will result in a detectable tissue. The tissues are
20 detected by visualization either without aid from a detector more sensitive than the human eye, or with the use of a light source to excite any fluorescent products.

As used herein, visualizable means detectable by eye, particularly during surgery under normal surgical conditions, or, if necessary, slightly dimmed light.

25 As used herein, pharmaceutically acceptable salts, esters or other derivatives of the conjugates include any salts, esters or derivatives that may be readily prepared by those of skill in this art using known methods for such derivatization and that produce compounds that may be administered to animals or humans without substantial toxic effects and that either are pharmaceutically
30 active or are prodrugs.

As used herein, treatment means any manner in which the symptoms of a conditions, disorder or disease are ameliorated or otherwise beneficially

-38-

altered. Treatment also encompasses any pharmaceutical use of the compositions herein.

As used herein, amelioration of the symptoms of a particular disorder by administration of a particular pharmaceutical composition refers to any lessening, whether permanent or temporary, lasting or transient that can be attributed to or associated with administration of the composition.

As used herein, substantially pure means sufficiently homogeneous to appear free of readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis and high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially chemically pure compounds are known to those of skill in the art. A substantially chemically pure compound may, however, be a mixture of stereoisomers or isomers. In such instances, further purification might increase the specific activity of the compound.

As used herein, a prodrug is a compound that, upon in vivo administration, is metabolized or otherwise converted to the biologically, pharmaceutically or therapeutically active form of the compound. To produce a prodrug, the pharmaceutically active compound is modified such that the active compound will be regenerated by metabolic processes. The prodrug may be designed to alter the metabolic stability or the transport characteristics of a drug, to mask side effects or toxicity, to improve the flavor of a drug or to alter other characteristics or properties of a drug. By virtue of knowledge of pharmacodynamic processes and drug metabolism in vivo, those of skill in this art, once a pharmaceutically active compound is known, can design prodrugs of the compound (see, e.g., Nogrady (1985) Medicinal Chemistry A Biochemical Approach, Oxford University Press, New York, pages 388-392).

As used herein, biological activity refers to the in vivo activities of a compound or physiological responses that result upon in vivo administration of a compound, composition or other mixture. Biological activity, thus,

-39-

encompasses therapeutic effects and pharmaceutical activity of such compounds, compositions and mixtures. Biological activities may be observed in in vitro systems designed to test or use such activities. Thus, for purposes herein the biological activity of a luciferase is its oxygenase activity whereby,
5 upon oxidation of a substrate, light is produced.

As used herein, targeting agent refers to an agent that specifically or preferentially targets a linked targeted agent, a luciferin or luciferase, to a neoplastic cell or tissue.

As used herein, tumor antigen refers to a cell surface protein expressed
10 or located on the surface of tumor cells.

As used herein, neoplastic cells include any type of transformed or altered cell that exhibits characteristics typical of transformed cells, such as a lack of contact inhibition and the acquisition of tumor-specific antigens. Such cells include, but are not limited to leukemic cells and cells derived from a
15 tumor.

As used herein, neoplastic disease is any disease in which neoplastic cells are present in the individual afflicted with the disease. Such diseases include, any disease characterized as cancer.

As used herein, metastatic tumors refers to tumors that are not localized
20 in one site.

As used herein, specialty tissue refers to non-tumorous tissue for which information regarding location is desired. Such tissues include, for example, endometriotic tissue, ectopic pregnancies, tissues associated with certain disorders and myopathies or pathologies.

As used herein, a receptor refers to a molecule that has an affinity for a
25 given ligand. Receptors may be naturally-occurring or synthetic molecules. Receptors may also be referred to in the art as anti-ligands. As used herein, the receptor and anti-ligand are interchangeable. Receptors can be used in their unaltered state or as aggregates with other species. Receptors may be
30 attached, covalently or noncovalently, or in physical contact with, to a binding member, either directly or indirectly via a specific binding substance or linker. Examples of receptors, include, but are not limited to: antibodies, cell

-40-

membrane receptors surface receptors and internalizing receptors, monoclonal antibodies and antisera reactive with specific antigenic determinants [such as on viruses, cells, or other materials], drugs, polynucleotides, nucleic acids, peptides, cofactors, lectins, sugars, polysaccharides, cells, cellular membranes, and organelles.

Examples of receptors and applications using such receptors, include but are not restricted to:

- a) enzymes: specific transport proteins or enzymes essential to survival of microorganisms, which could serve as targets for antibiotic [ligand] selection;
- 10 b) antibodies: identification of a ligand-binding site on the antibody molecule that combines with the epitope of an antigen of interest may be investigated; determination of a sequence that mimics an antigenic epitope may lead to the development of vaccines of which the immunogen is based on one or more of such sequences or lead to the development of related diagnostic
- 15 agents or compounds useful in therapeutic treatments such as for auto-immune diseases
- c) nucleic acids: identification of ligand, such as protein or RNA, binding sites;
- d) catalytic polypeptides: polymers, preferably polypeptides, that are
- 20 capable of promoting a chemical reaction involving the conversion of one or more reactants to one or more products; such polypeptides generally include a binding site specific for at least one reactant or reaction intermediate and an active functionality proximate to the binding site, in which the functionality is capable of chemically modifying the bound reactant [see, e.g., U.S. Patent No.
- 25 5,215,899];
- e) hormone receptors: determination of the ligands that bind with high affinity to a receptor is useful in the development of hormone replacement therapies; for example, identification of ligands that bind to such receptors may lead to the development of drugs to control blood pressure; and
- 30 f) opiate receptors: determination of ligands that bind to the opiate receptors in the brain is useful in the development of less-addictive replacements for morphine and related drugs.

-41-

As used herein, antibody includes antibody fragments, such as Fab fragments, which are composed of a light chain and the variable region of a heavy chain.

As used herein, an antibody conjugate refers to a conjugate in which the
5 targeting agent is an antibody.

As used herein, antibody activation refers to the process whereby activated antibodies are produced. Antibodies are activated upon reaction with a linker, such as heterobifunctional reagent.

As used herein, a surgical viewing refers to any procedure in which an
10 opening is made in the body of an animal. Such procedures include traditional surgeries and diagnostic procedures, such as laparoscopies and arthroscopic procedures.

As used herein, humanized antibodies refer to antibodies that are modified to include "human" sequences of amino acids so that administration to
15 a human will not provoke an immune response. Methods for preparation of such antibodies are known. For example, the hybridoma that expresses the monoclonal antibody is altered by recombinant DNA techniques to express an antibody in which the amino acid composition of the non-variable regions is based on human antibodies. Computer programs have been designed to identify
20 such regions.

As used herein, ATP, AMP, NAD⁺ and NADH refer to adenosine triphosphate, adenosine monophosphate, nicotinamide adenine dinucleotide (oxidized form) and nicotinamide adenine dinucleotide (reduced form), respectively.

As used herein, production by recombinant means by using recombinant
25 DNA methods means the use of the well known methods of molecular biology for expressing proteins encoded by cloned DNA.

As used herein, substantially identical to a product means sufficiently similar so that the property of interest is sufficiently unchanged so that the
30 substantially identical product can be used in place of the product.

As used herein equivalent, when referring to two sequences of nucleic acids means that the two sequences in question encode the same sequence of

-42-

amino acids or equivalent proteins. When "equivalent" is used in referring to two proteins or peptides, it means that the two proteins or peptides have substantially the same amino acid sequence with only conservative amino acid substitutions [see, e.g., Table 1, above] that do not substantially alter the activity or function of the protein or peptide. When "equivalent" refers to a property, the property does not need to be present to the same extent [e.g., two peptides can exhibit different rates of the same type of enzymatic activity], but the activities are preferably substantially the same. "Complementary," when referring to two nucleotide sequences, means that the two sequences of nucleotides are capable of hybridizing, preferably with less than 25%, more preferably with less than 15%, even more preferably with less than 5%, most preferably with no mismatches between opposed nucleotides. Preferably the two molecules will hybridize under conditions of high stringency.

As used herein: stringency of hybridization in determining percentage mismatch is as follows:

- 1) high stringency: 0.1 x SSPE, 0.1% SDS, 65°C
- 2) medium stringency: 0.2 x SSPE, 0.1% SDS, 50°C
- 3) low stringency: 1.0 x SSPE, 0.1% SDS, 50°C

It is understood that equivalent stringencies may be achieved using alternative buffers, salts and temperatures.

The term "substantially" identical or homologous or similar varies with the context as understood by those skilled in the relevant art and generally means at least 70%, preferably means at least 80%, more preferably at least 90%, and most preferably at least 95% identity.

As used herein, a composition refers to a any mixture. It may be a solution, a suspension, liquid, powder, a paste, aqueous, non-aqueous or any combination thereof.

As used herein, a combination refers to any association between two or among more items.

As used herein, fluid refers to any composition that can flow. Fluids thus encompass compositions that are in the form of semi-solids, pastes, solutions, aqueous mixtures, gels, lotions, creams and other such compositions.

-43-

Examples of receptors and applications using such receptors, include but are not restricted to:

- a) enzymes: specific transport proteins or enzymes essential to survival of microorganisms, which could serve as targets for antibiotic [ligand] selection;
- 5 b) antibodies: identification of a ligand-binding site on the antibody molecule that combines with the epitope of an antigen of interest may be investigated; determination of a sequence that mimics an antigenic epitope may lead to the development of vaccines of which the immunogen is based on one or more of such sequences or lead to the development of related diagnostic
10 agents or compounds useful in therapeutic treatments such as for auto-immune diseases
- c) nucleic acids: identification of ligand, such as protein or RNA, binding sites;
- d) catalytic polypeptides: polymers, preferably polypeptides, that are
15 capable of promoting a chemical reaction involving the conversion of one or more reactants to one or more products; such polypeptides generally include a binding site specific for at least one reactant or reaction intermediate and an active functionality proximate to the binding site, in which the functionality is capable of chemically modifying the bound reactant [see, e.g., U.S. Patent No.
20 5,215,899];
- e) hormone receptors: determination of the ligands that bind with high affinity to a receptor is useful in the development of hormone replacement therapies; for example, identification of ligands that bind to such receptors may lead to the development of drugs to control blood pressure; and
- 25 f) opiate receptors: determination of ligands that bind to the opiate receptors in the brain is useful in the development of less-addictive replacements for morphine and related drugs.

As used herein, complementary refers to the topological compatibility or matching together of interacting surfaces of a ligand molecule and its receptor.
30 Thus, the receptor and its ligand can be described as complementary, and furthermore, the contact surface characteristics are complementary to each other.

-44-

As used herein, a ligand-receptor pair or complex formed when two macromolecules have combined through molecular recognition to form a complex.

As used herein, a substrate refers to any matrix that is used either
5 directly or following suitable derivatization, as a solid support for chemical synthesis, assays and other such processes. Preferred substrates herein, are silicon substrates or siliconized substrates that are derivitized on the surface intended for linkage of anti-ligands and ligands and other macromolecules, including the fluorescent proteins, phycobiliproteins and other emission shifters.

10 As used herein, a matrix refers to any solid or semisolid or insoluble support on which the molecule of interest, typically a biological molecule, macromolecule, organic molecule or biospecific ligand is linked or contacted. Typically a matrix is a substrate material having a rigid or semi-rigid surface. In many embodiments, at least one surface of the substrate will be substantially
15 flat, although in some embodiments it may be desirable to physically separate synthesis regions for different polymers with, for example, wells, raised regions, etched trenches, or other such topology. Matrix materials include any materials that are used as affinity matrices or supports for chemical and biological molecule syntheses and analyses, such as, but are not limited to: polystyrene,
20 polycarbonate, polypropylene, nylon, glass, dextran, chitin, sand, pumice, polytetrafluoroethylene, agarose, polysaccharides, dendrimers, buckyballs, polyacrylamide, Kieselguhr-polyacrylamide non-covalent composite, polystyrene-polyacrylamide covalent composite, polystyrene-PEG [polyethyleneglycol] composite, silicon, rubber, and other materials used as supports for solid phase
25 syntheses, affinity separations and purifications, hybridization reactions, immunoassays and other such applications.

As used herein, the attachment layer refers the surface of the chip device to which molecules are linked. Typically, the chip is a semiconductor device, which is coated on at least a portion of the surface to render it suitable
30 for linking molecules and inert to any reactions to which the device is exposed. Molecules are linked either directly or indirectly to the surface, linkage may be effected by absorption or adsorption, through covalent bonds, ionic interactions

-45-

or any other interaction. Where necessary the attachment layer is adapted, such as by derivatization for linking the molecules.

B. BIOLUMINESCENCE GENERATING SYSTEMS AND COMPONENTS

The following is a description of bioluminescence generating systems and the components thereof. These luciferases and luciferins and fluorescent proteins can be used with the luciferases and GFPs provided herein.

1. Exemplary bioluminescence generating systems

A bioluminescence-generating system refers to the components that are necessary and sufficient to generate bioluminescence. These include a luciferase, luciferin and any necessary co-factors or conditions. Virtually any bioluminescent system known to those of skill in the art will be amenable to use in the apparatus, systems, combinations and methods provided herein. Factors for consideration in selecting a bioluminescent-generating system, include, but are not limited to: the targeting agent used in combination with the bioluminescence; the medium in which the reaction is run; stability of the components, such as temperature or pH sensitivity; shelf life of the components; sustainability of the light emission, whether constant or intermittent; availability of components; desired light intensity; color of the light; and other such factors.

a. General description

In general, bioluminescence refers to an energy-yielding chemical reaction in which a specific chemical substrate, a luciferin, undergoes oxidation, catalyzed by an enzyme, a luciferase. Bioluminescent reactions are easily maintained, requiring only replenishment of exhausted luciferin or other substrate or cofactor or other protein, in order to continue or revive the reaction. Bioluminescence generating reactions are well-known to those of skill in this art and any such reaction may be adapted for use in combination with articles of manufacture as described herein.

There are numerous organisms and sources of bioluminescence generating systems, and some representative genera and species that exhibit bioluminescence are set forth in the following table [reproduced in part from

-46-

Hastings in (1995) *Cell Physiology:Source Book*, N. Sperelakis (ed.), Academic Press, pp 665-681]:

TABLE 2
Representative luminous organism

	Type of Organism	Representative genera
	Bacteria	Photobacterium Vibrio Xenorhabdus
	Mushrooms	Panus, Armillaria Pleurotus
	Dinoflagellates	Gonyaulax Pyrocystis Noctiluca
10	Cnidaria (coelenterates) Jellyfish Hydroid Sea Pansy	Aequorea Obelia Renilla
	Ctenophores	Mnemiopsis Beroe
15	Annelids Earthworms Marine polychaetes Syllid fireworm	Diplocardia Chaetopterus, Phyxotrix Odontosyllis
20	Molluscs Limpet Clam Squid	Latia Pholas Heteroteuthis Heterocarpus
	Crustacea Ostracod	Vargula (Cypridina)
25	Shrimp (euphausids)	Meganyctiphanes Acanthophyra Oplophorus Gnathophausia
30	Decapod Copepods	Sergestes

-47-

	Type of Organism	Representative genera
5	Insects Coleopterids (beetles) Firefly Click beetles Railroad worm Diptera (flies)	Photinus, Photuris Pyrophorus Phengodes, Phrixothrix Arachnocampa
10	Echinoderms Brittle stars Sea cucumbers	Ophiopsila Laetmogone
15	Chordates Tunicates	Pyrosoma
20	Fish Cartilaginous Bony Ponyfish Flashlight fish Angler fish Midshipman Lantern fish Shiny loosejaw Hatchet fish and other fish Midwater fish	Squalus Leiognathus Photoblepharon Cryptopsaras Porichthys Benia Aristostomias Agyropelecus Pachystomias Malacosteus Cyclothone Neoscopelus Tarletonbeania

25 Other bioluminescent organisms contemplated for use herein are *Gonadostomias*, *Gaussia* (copepods), *Watensia*, *Halisturia*, Vampire squid, *Glyphus*, Mycotophids (fish), *Vinciguerria*, *Howella*, *Florenciella*, *Chaudiodus*, *Melanocostus* and Sea Pens.

30 It is understood that a bioluminescence generating system may be isolated from natural sources, such as those in the above Table, or may be produced synthetically. In addition, for uses herein, the components need only be sufficiently pure so that mixture thereof, under appropriate reaction conditions, produces a glow so that cells and tissues can be visualized during a surgical procedure.

-48-

Thus, in some embodiments, a crude extract or merely grinding up the organism may be adequate. Generally, however, substantially pure components are used. Also, components may be synthetic components that are not isolated from natural sources. DNA encoding luciferases is available [see, e.g., SEQ ID
5 Nos. 1-13] and has been modified [see, e.g., SEQ ID Nos. 3 and 10-13] and synthetic and alternative substrates have been devised. The DNA listed herein is only representative of the DNA encoding luciferases that is available.

Any bioluminescence generating system, whether synthetic or isolated from natural sources, such as those set forth in Table 2, elsewhere herein or
10 known to those of skill in the art, is intended for use in the combinations, systems and methods provided herein. Chemiluminescence systems per se, which do not rely on oxygenases [luciferases] are not encompassed herein.

(1) Luciferases

The targeted agents herein include luciferases or luciferins. Luciferases
15 refer to any compound that, in the presence of any necessary activators, catalyze the oxidation of a bioluminescence substrate [luciferin] in the presence of molecular oxygen, whether free or bound, from a lower energy state to a higher energy state such that the substrate, upon return to the lower energy state, emits light. For purposes herein, luciferase is broadly used to encompass
20 enzymes that act catalytically to generate light by oxidation of a substrate and also photoproteins, such as aequorin, that act, though not strictly catalytically [since such proteins are exhausted in the reaction], in conjunction with a substrate in the presence of oxygen to generate light. These luciferases, including photoproteins, such as aequorin, are herein also included among the
25 luciferases. These reagents include the naturally-occurring luciferases [including photoproteins], proteins produced by recombinant DNA, and mutated or modified variants thereof that retain the ability to generate light in the presence of an appropriate substrate, co-factors and activators or any other such protein that acts as a catalyst to oxidize a substrate, whereby light is produced.

30 Generically, the protein that catalyzes or initiates the bioluminescent reaction is referred to as a luciferase, and the oxidizable substrate is referred to as a luciferin. The oxidized reaction product is termed oxyluciferin, and certain

-49-

luciferin precursors are termed etioluciferin. Thus, for purposes herein bioluminescence encompasses light produced by reactions that are catalyzed by [in the case of luciferases that act enzymatically] or initiated by [in the case of the photoproteins, such as aequorin, that are not regenerated in the reaction] a
5 biological protein or analog, derivative or mutant thereof.

For clarity herein, these catalytic proteins are referred to as luciferases and include enzymes such as the luciferases that catalyze the oxidation of luciferin, emitting light and releasing oxyluciferin. Also included among luciferases are photoproteins, which catalyze the oxidation of luciferin to emit
10 light but are changed in the reaction and must be reconstituted to be used again. The luciferases may be naturally occurring or may be modified, such as by genetic engineering to improve or alter certain properties. As long as the resulting molecule retains the ability to catalyze the bioluminescent reaction, it is encompassed herein.

15 Any protein that has luciferase activity [a protein that catalyzes oxidation of a substrate in the presence of molecular oxygen to produce light as defined herein] may be used herein. The preferred luciferases are those that are described herein or that have minor sequence variations. Such minor sequence variations include, but are not limited to, minor allelic or species variations and
20 insertions or deletions of residues, particularly cysteine residues. Suitable conservative substitutions of amino acids are known to those of skill in this art and may be made generally without altering the biological activity of the resulting molecule. Such substitutions are preferably made in accordance with those set forth in TABLE 1 as described above.

25 The luciferases may be obtained commercially, isolated from natural sources, expressed in host cells using DNA encoding the luciferase, or obtained in any manner known to those of skill in the art. For purposes herein, crude extracts obtained by grinding up selected source organisms may suffice. Since large quantities of the luciferase may be desired, isolation of the luciferase from
30 host cells is preferred. DNA for such purposes is widely available as are modified forms thereof.

-50-

Examples of luciferases include, but are not limited to, those isolated from the ctenophores *Mnemiopsis* (mnemiopsin) and *Beroe ovata* (berovin), those isolated from the coelenterates *Aequorea* (aequorin), *Obelia* (obelin), *Pelagia*, the *Renilla* luciferase, the luciferases isolated from the mollusca *Pholas* (pholasin), the luciferases isolated from fish, such as *Aristostomias*, *Pachystomias* and *Porichthys* and from the ostracods, such as *Cypridina* (also referred to as *Vargula*). Preferred luciferases for use herein are the Aequorin protein, *Renilla* luciferase and *Cypridina* [also called *Vargula*] luciferase [see, e.g., SEQ ID Nos. 1, 2, and 4-13]. Also, preferred are luciferases which react to produce red and/or near infrared light. These include luciferases found in species of *Aristostomias*, such as *A. scintillans*, *Pachystomias*, *Malacosteus*, such as *M. niger*.

(2) Luciferins

The substrates for the reaction or for inclusion in the conjugates include any molecule(s) with which the luciferase reacts to produce light. Such molecules include the naturally-occurring substrates, modified forms thereof, and synthetic substrates [see, e.g., U.S. Patent Nos. 5,374,534 and 5,098,828]. Exemplary luciferins include those described herein, as well as derivatives thereof, analogs thereof, synthetic substrates, such as dioxetanes [see, e.g., U.S. Patent Nos. 5,004,565 and 5,455,357], and other compounds that are oxidized by a luciferase in a light-producing reaction [see, e.g., U.S. Patent Nos. 5,374,534, 5,098,828 and 4,950,588]. Such substrates also may be identified empirically by selecting compounds that are oxidized in bioluminescent reactions.

(3) Activators

The bioluminescent generating systems also require additional components discussed herein and known to those of skill in the art. All bioluminescent reactions require molecular oxygen in the form of dissolved or bound oxygen. Thus, molecular oxygen, dissolved in water or in air or bound to a photoprotein, is the activator for bioluminescence reactions. Depending upon the form of the components, other activators include, but are not limited to,

-51-

ATP [for firefly luciferase], flavin reductase [bacterial systems] for regenerating FMNH₂ from FMN, and Ca²⁺ or other suitable metal ion [aequorin].

Most of the systems provided herein will generate light when the luciferase and luciferin are mixed and exposed to air or water. The systems that
5 use photoproteins that have bound oxygen, such as aequorin, however, will require exposure to Ca²⁺ [or other suitable metal ion], which can be provided in the form of an aqueous composition of a calcium salt. In these instances, addition of a Ca²⁺ [or other suitable metal ion] to a mixture of luciferase [aequorin] and luciferin [such as coelenterazine] will result in generation of light.
10 The *Renilla* system and other Anthozoa systems also require Ca²⁺ [or other suitable metal ion].

If crude preparations are used, such as ground up *Cypridina* [shrimp] or ground fireflies, it may be necessary to add only water. In instances in which fireflies [or a firefly or beetle luciferase] are used the reaction may only require
15 addition ATP. The precise components will be apparent, in light of the disclosure herein, to those of skill in this art or may be readily determined empirically.

It is also understood that these mixtures will also contain any additional salts or buffers or ions that are necessary for each reaction to proceed. Since
20 these reactions are well-characterized, those of skill in the art will be able to determine precise proportions and requisite components. Selection of components will depend upon the apparatus, article of manufacture and luciferase. Various embodiments are described and exemplified herein; in view of such description, other embodiments will be apparent.

25 (4) Reactions

In all embodiments, all but one component, either the luciferase or luciferin, of a bioluminescence generating system will be mixed or packaged with or otherwise combined. The remaining component is conjugated to a targeting agent and is intended for administration to an animal.

30 Prior to a surgical procedure, the conjugate is administered via any suitable route, whereby the targeting agent binds to the targeted tissue by virtue of its specific interaction with a tissue-specific cell surface protein.

-52-

During surgery the tissue is contacted, with the remaining component(s), typically by spraying the area or local injection, and any tissue to which conjugate is bound will glow. The glow should be sufficient to see under dim light or, if necessary, in the dark.

- 5 In general, since the result to be achieved is the production of light visible to the naked eye for qualitative, not quantitative, diagnostic purposes, the precise proportions and amounts of components of the bioluminescence reaction need not be stringently determined or met. They must be sufficient to produce light. Generally, an amount of luciferin and luciferase sufficient to
- 10 generate a visible glow is used; this amount can be readily determined empirically and is dependent upon the selected system and selected application. Where quantitative measurements are required, more precision may be required.
- For purposes herein, such amount is preferably at least the concentrations and proportions used for analytical purposes by those of skill in
- 15 the such arts. Higher concentrations may be used if the glow is not sufficiently bright. Alternatively, a microcarrier coupled to more than one luciferase molecule linked to a targeting agent may be utilized to increase signal output. Also because the conditions in which the reactions are used are not laboratory conditions and the components are subject to storage, higher concentration may
- 20 be used to overcome any loss of activity. Typically, the amounts are 1 mg, preferably 10 mg and more preferably 100 mg, of a luciferase per liter of reaction mixture or 1 mg, preferably 10 mg, more preferably 100 mg. Compositions may contain at least about 0.01 mg/l, and typically 0.1 mg/l, 1 mg/l, 10 mg/l or more of each component on the item. The amount of luciferin
- 25 is also between about 0.01 and 100 mg/l, preferably between 0.1 and 10 mg/l, additional luciferin can be added to many of the reactions to continue the reaction. In embodiments in which the luciferase acts catalytically and does not need to be regenerated, lower amounts of luciferase can be used. In those in which it is changed during the reaction, it also can be replenished; typically
- 30 higher concentrations will be selected. Ranges of concentration per liter [or the amount of coating on substrate the results from contacting with such composition] of each component on the order of 0.1 to 20 mg, preferably 0.1 to

-53-

10 mg, more preferably between about 1 and 10 mg of each component will be sufficient. When preparing coated substrates, as described herein, greater amounts of coating compositions containing higher concentrations of the luciferase or luciferin may be used.

- 5 Thus, for example, in presence of calcium, 5 mg of luciferin, such as coelenterazine, in one liter of water will glow brightly for at least about 10 to 20 minutes, depending on the temperature of the water, when about 10 mgs of luciferase, such as aequorin photoprotein luciferase or luciferase from *Renilla*, is added thereto. Increasing the concentration of luciferase, for example, to 100
10 mg/l, provides a particularly brilliant display of light.

- It is understood, that concentrations and amounts to be used depend upon the selected bioluminescence generating system but these may be readily determined empirically. Proportions, particularly those used when commencing an empirical determination, are generally those used for analytical purposes, and
15 amounts or concentrations are at least those used for analytical purposes, but the amounts can be increased, particularly if a sustained and brighter glow is desired.

2. The *Renilla* system

- Renilla*, also known as soft coral sea pansies, are members of the class
20 of coelenterates Anthozoa, which includes other bioluminescent genera, such as *Cavarnularia*, *Ptilosarcus*, *Stylatula*, *Acanthoptilum*, and *Parazoanthus*. Bioluminescent members of the Anthozoa genera contain luciferases and luciferins that are similar in structure [see, e.g., Cormier *et al.* (1973) *J. Cell. Physiol.* 81:291-298; see, also Ward *et al.* (1975) *Proc. Natl. Acad. Sci. U.S.A.*
25 *72*:2530-2534]. The luciferases and luciferins from each of these anthozoans crossreact with one another and produce a characteristic blue luminescence.

- Renilla* luciferase and the other coelenterate and ctenophore luciferases, such as the aequorin photoprotein, use imidazopyrazine substrates, particularly the substrates generically called coelenterazine [see, formulae (I) and (II) of
30 Section B.1.b, above]. Other genera that have luciferases that use a coelenterazine include: squid, such as *Chiroteuthis*, *Eucleoteuthis*, *Onychoteuthis*, *Watasenia*, cuttlefish, *Sepiolina*; shrimp, such as *Oplophorus*,

-54-

Acanthophyra, *Sergestes*, and *Gnathophausia*; deep-sea fish, such as *Argyroteleus*, *Yarella*, *Diaphus*, *Gonadostomias* and *Neoscopelus*.

Renilla luciferase does not, however, have bound oxygen, and thus requires dissolved oxygen in order to produce light in the presence of a suitable luciferin substrate. Since *Renilla* luciferase acts as a true enzyme [i.e., it does not have to be reconstituted for further use] the resulting luminescence can be long-lasting in the presence of saturating levels of luciferin. Also, *Renilla* luciferase is relatively stable to heat.

Renilla luciferases, DNA encoding *Renilla reniformis* luciferase, and use of the *Renilla reniformis* DNA to produce recombinant luciferase, as well as DNA encoding luciferase from other coelenterates, are well known and available [see, e.g., SEQ ID No. 1, U.S. Patent Nos. 5,418,155 and 5,292,658; see, also, Prasher et al. (1985) Biochem. Biophys. Res. Commun. 126:1259-1268; Cormier (1981) "Renilla and Aequorea bioluminescence" in Bioluminescence and Chemiluminescence, pp. 225-233; Charbonneau et al. (1979) J. Biol. Chem. 254:769-780; Ward et al. (1979) J. Biol. Chem. 254:781-788; Lorenz et al. (1981) Proc. Natl. Acad. Sci. U.S.A. 88: 4438-4442; Hori et al. (1977) Proc. Natl. Acad. Sci. U.S.A. 74:4285-4287; Hori et al. (1975) Biochemistry 14:2371-2376; Hori et al. (1977) Proc. Natl. Acad. Sci. U.S.A. 74:4285-4287; Inouye et al. (1975) Jap. Soc. Chem. Lett. 141-144; and Matthews et al. (1979) Biochemistry 16:85-91]. The DNA encoding *Renilla reniformis* luciferase and host cells containing such DNA provide a convenient means for producing large quantities of *Renilla reniformis* enzyme, such as in those known to those of skill in the art [see, e.g., U.S. Patent Nos. 5,418,155 and 5,292,658, which describe recombinant production of *Renilla reniformis* luciferase].

When used herein, the *Renilla* luciferase can be packaged in lyophilized form, encapsulated in a vehicle, either by itself or in combination with the luciferin substrate. Prior to use the mixture is contacted with an aqueous composition, preferably a phosphate buffered saline pH 7-8; dissolved O₂ will activate the reaction. Final concentrations of luciferase in the glowing mixture will be on the order of 0.01 to 1 mg/l or more. Concentrations of luciferin will

-55-

be at least about 10^{-8} M, but 1 to 100 or more orders of magnitude higher to produce a long lasting bioluminescence.

In certain embodiments herein, about 1 to 10 mg, or preferably 2-5 mg, more preferably about 3 mg of coelenterazine will be used with about 100 mg
5 of *Renilla* luciferase. The precise amounts, of course can be determined empirically, and, also will depend to some extent on the ultimate concentration and application. In particular, about addition of about 0.25 ml of a crude extract from the bacteria that express *Renilla* to 100 ml of a suitable assay buffer and about 0.005 μ g was sufficient to produce a visible and lasting glow
10 [see, U.S. Patent Nos. 5,418,155 and 5,292,658, which describe recombinant production of *Renilla reniformis* luciferase].

Lyophilized mixtures, and compositions containing the *Renilla* luciferase are also provided. The luciferase or mixtures of the luciferase and luciferin may also be encapsulated into a suitable delivery vehicle, such as a liposome, glass
15 particle, capillary tube, drug delivery vehicle, gelatin, time release coating or other such vehicle. The luciferase may also be linked to a substrate, such as biocompatible materials.

b. Ctenophore systems

Ctenophores, such as *Mnemiopsis* (mnemiopsin) and *Beroe ovata*
20 (berovin), and coelenterates, such as *Aequorea* (aequorin), *Obelia* (obelin) and *Pelagia*, produce bioluminescent light using similar chemistries [see, e.g., Stephenson et al. (1981) Biochimica et Biophysica Acta 678:65-75; Hart et al. (1979) Biochemistry 18:2204-2210; International PCT Application No. WO 94/18342, which is based on U.S. application Serial No. 08/017,116, U.S.
25 Patent No. 5,486,455 and other references and patents cited herein]. The *Aequorin* and *Renilla* systems are representative and are described in detail herein as exemplary and as among the presently preferred systems. The *Aequorin* and *Renilla* systems can use the same luciferin and produce light using the same chemistry, but each luciferase is different. The *Aequorin* luciferase
30 aequorin, as well as, for example, the luciferases mnemiopsin and berovin, is a photoprotein that includes bound oxygen and bound luciferin, requires Ca^{2+} [or other suitable metal ion] to trigger the reaction, and must be regenerated for

-56-

repeated use; whereas, the *Renilla* luciferase acts as a true enzyme because it is unchanged during the reaction and it requires dissolved molecular oxygen.

(1) The aequorin system

The aequorin system is well known [see, e.g., Tsuji *et al.* (1986)

- 5 "Site-specific mutagenesis of the calcium-binding photoprotein aequorin," Proc. Natl. Acad. Sci. USA 83:8107-8111; Prasher *et al.* (1985) "Cloning and Expression of the cDNA Coding for Aequorin, a Bioluminescent Calcium-Binding Protein," Biochemical and Biophysical Research Communications 126:1259-1268; Prasher *et al.* (1986) Methods in Enzymology 133:288-297;
- 10 Prasher, *et al.* (1987) "Sequence Comparisons of cDNAs Encoding for Aequorin Isotypes," Biochemistry 26:1326-1332; Charbonneau *et al.* (1985) "Amino Acid Sequence of the Calcium-Dependent Photoprotein Aequorin," Biochemistry 24:6762-6771; Shimomura *et al.* (1981) "Resistivity to denaturation of the apoprotein of aequorin and reconstitution of the luminescent
- 15 photoprotein from the partially denatured apoprotein," Biochem. J. 199:825-828; Inouye *et al.* (1989) J. Biochem. 105:473-477; Inouye *et al.* (1986) "Expression of Apoequorin Complementary DNA in *Escherichia coli*," Biochemistry 25:8425-8429; Inouye *et al.* (1985) "Cloning and sequence analysis of cDNA for the luminescent protein aequorin," Proc. Natl. Acad. Sci.
- 20 USA 82:3154-3158; Prendergast, *et al.* (1978) "Chemical and Physical Properties of Aequorin and the Green Fluorescent Protein Isolated from *Aequorea forskalea*" J. Am. Chem. Soc. 100:3448-3453; European Patent Application 0 540 064 A1; European Patent Application 0 226 979 A2, European Patent Application 0 245 093 A1 and European Patent Application 0
- 25 245 093 B1; U.S. Patent No. 5,093,240; U.S. Patent No. 5,360,728; U.S. Patent No. 5,139,937; U.S. Patent No. 5,422,266; U.S. Patent No. 5,023,181; U.S. Patent No. 5,162,227; and SEQ ID Nos. 5-13, which set forth DNA encoding the apoprotein; and a form, described in U.S. Patent No. 5,162,227, European Patent Application 0 540 064 A1 and Sealite Sciences Technical
- 30 Report No. 3 (1994), is commercially available from Sealite, Sciences, Bogart, GA as AQUALITE®].

-57-

This system is among the preferred systems for use herein. As will be evident, since the aequorin photoprotein includes noncovalently bound luciferin and molecular oxygen, it is suitable for storage in this form as a lyophilized powder or encapsulated into a selected delivery vehicle. The system can be
5 encapsulated into pellets, such as liposomes or other delivery vehicles. When used, the vehicles are contacted with a composition, even tap water, that contains Ca^{2+} [or other suitable metal ion], to produce a mixture that glows.

(a) **Aequorin and related photoproteins**

The photoprotein, aequorin, isolated from the jellyfish, *Aequorea*, emits
10 light upon the addition of Ca^{2+} [or other suitable metal ion]. The aequorin photoprotein, which includes bound luciferin and bound oxygen that is released by Ca^{2+} , does not require dissolved oxygen. Luminescence is triggered by calcium, which releases oxygen and the luciferin substrate producing apoaequorin.

15 The bioluminescence photoprotein aequorin is isolated from a number of species of the jellyfish *Aequorea*. It is a 22 kilodalton [kD] molecular weight peptide complex [see, e.g., Shimomura *et al.* (1962) *J. Cellular and Comp. Physiol.* 59:233-238; Shimomura *et al.* (1969) *Biochemistry* 8:3991-3997; Kohama *et al.* (1971) *Biochemistry* 10:4149-4152; and Shimomura *et al.* (1972)
20 *Biochemistry* 11:1602-1608]. The native protein contains oxygen and a heterocyclic compound coelenterazine, a luciferin, [see, below] noncovalently bound thereto. The protein contains three calcium binding sites. Upon addition of trace amounts Ca^{2+} [or other suitable metal ion, such as strontium] to the photoprotein, it undergoes a conformational change that catalyzes the oxidation
25 of the bound coelenterazine using the protein-bound oxygen. Energy from this oxidation is released as a flash of blue light, centered at 469 nm. Concentrations of calcium ions as low as 10^{-6} M are sufficient to trigger the oxidation reaction.

Naturally-occurring apoaequorin is not a single compound but rather is a
30 mixture of microheterogeneous molecular species. *Aequoria* jellyfish extracts contain as many as twelve distinct variants of the protein [see, e.g., Prasher *et al.* (1987) *Biochemistry* 26:1326-1332; Blinks *et al.* (1975) *Fed. Proc.* 34:474].

-58-

DNA encoding numerous forms has been isolated [see, e.g., SEQ ID Nos. 5-9 and 13].

The photoprotein can be reconstituted [see, e.g., U.S. Patent No. 5,023,181] by combining the apoprotein, such as a protein recombinantly produced in E. coli, with a coelenterazine, such as a synthetic coelenterazine, in the presence of oxygen and a reducing agent [see, e.g., Shimomura et al. (1975) Nature 256:236-238; Shimomura et al. (1981) Biochemistry J. 199:825-828], such as 2-mercaptoethanol, and also EDTA or EGTA [concentrations between about 5 to about 100 mM or higher for applications herein] tie up any Ca^{2+} to prevent triggering the oxidation reaction until desired. DNA encoding a modified form of the apoprotein that does not require 2-mercaptoethanol for reconstitution is also available [see, e.g., U.S. Patent No. U.S. Patent No. 5,093,240]. The reconstituted photoprotein is also commercially available [sold, e.g., under the trademark AQUALITE[®], which is described in U.S. Patent No. 5,162,227].

The light reaction is triggered by adding Ca^{2+} at a concentration sufficient to overcome the effects of the chelator and achieve the 10^{-6} M concentration. Because such low concentrations of Ca^{2+} can trigger the reaction, for use in the methods herein, higher concentrations of chelator may be included in the compositions of photoprotein. Accordingly, higher concentrations of added Ca^{2+} in the form of a calcium salt will be required. Precise amounts may be empirically determined. For use herein, it may be sufficient to merely add water to the photoprotein, which is provided in the form of a concentrated composition or in lyophilized or powdered form. Thus, for purposes herein, addition of small quantities of Ca^{2+} , such as those present in phosphate buffered saline (PBS) or other suitable buffers or the moisture on the tissue to which the compositions are contacted, should trigger the bioluminescence reaction.

Numerous isoforms of the aequorin apoprotein been identified isolated. DNA encoding these proteins has been cloned, and the proteins and modified forms thereof have been produced using suitable host cells [see, e.g., U.S. Patent Nos. 5,162,227, 5,360,728, 5,093,240; see, also, Prasher et al. (1985)

-59-

Biophys. Biochem. Res. Commun. 126:1259-1268; Inouye et al. (1986) Biochemistry 25: 8425-8429]. U.S. Patent No. 5,093,240; U.S. Patent No. 5,360,728; U.S. Patent No. 5,139,937; U.S. Patent No. 5,288,623; U.S. Patent No. 5,422,266, U.S. Patent No. 5,162,227 and SEQ ID Nos. 5-13, which set forth DNA encoding the apoprotein; and a form is commercially available from Sealite, Sciences, Bogart, GA as AQUALITE®]. DNA encoding apoaequorin or variants thereof is useful for recombinant production of high quantities of the apoprotein. The photoprotein is reconstituted upon addition of the luciferin, coelenterazine, preferably a sulfated derivative thereof, or an analog thereof, and molecular oxygen [see, e.g., U.S. Patent No. 5,023,181]. The apoprotein and other constituents of the photoprotein and bioluminescence generating reaction can be mixed under appropriate conditions to regenerate the photoprotein and concomitantly have the photoprotein produce light. Reconstitution requires the presence of a reducing agent, such as mercaptoethanol, except for modified forms, discussed below, that are designed so that a reducing agent is not required [see, e.g., U.S. Patent No. 5,093,240].

For use herein, it is preferred aequorin is produced using DNA, such as that set forth in SEQ ID Nos. 5-13 and known to those of skill in the art or modified forms thereof. The DNA encoding aequorin is expressed in a host cell, such as E. coli, isolated and reconstituted to produce the photoprotein [see, e.g., U.S. Patent Nos. 5,418,155, 5,292,658, 5,360,728, 5,422,266, 5,162,227].

Of interest herein, are forms of the apoprotein that have been modified so that the bioluminescent activity is greater than unmodified apoaequorin [see, e.g., U.S. Patent No. 5,360,728, SEQ ID Nos. 10-12]. Modified forms that exhibit greater bioluminescent activity than unmodified apoaequorin include proteins including sequences set forth in SEQ ID Nos. 10-12, in which aspartate 124 is changed to serine, glutamate 135 is changed to serine, and glycine 129 is changed to alanine, respectively. Other modified forms with increased bioluminescence are also available.

For use in certain embodiments herein, the apoprotein and other components of the aequorin bioluminescence generating system are packaged

-60-

or provided as a mixture, which, when desired is subjected to conditions under which the photoprotein reconstitutes from the apoprotein, luciferin and oxygen [see, e.g., U.S. Patent No. 5,023,181; and U.S. Patent No. 5,093,240].

Particularly preferred are forms of the apoprotein that do not require a reducing agent, such as 2-mercaptoethanol, for reconstitution. These forms, described, for example in U.S. Patent No. 5,093,240 [see, also Tsuji et al. (1986) Proc. Natl. Acad. Sci. U.S.A. 83:8107-8111], are modified by replacement of one or more, preferably all three cysteine residues with, for example serine.

Replacement may be effected by modification of the DNA encoding the aequorin apoprotein, such as that set forth in SEQ ID No. 5, and replacing the cysteine codons with serine.

The photoproteins and luciferases from related species, such as *Obelia* are also contemplated for use herein. DNA encoding the Ca^{2+} -activated photoprotein obelin from the hydroid polyp *Obelia longissima* is known and available [see, e.g., Illarionov et al. (1995) Gene 153:273-274; and Bondar et al. (1995) Biochim. Biophys. Acta 1231:29-32]. This photoprotein can also be activated by Mn^{2+} [see, e.g., Vysotski et al. (1995) Arch. Bioch. Biophys. 316:92-93, Vysotski et al. (1993) J. Biolumin. Chemilumin. 8:301-305].

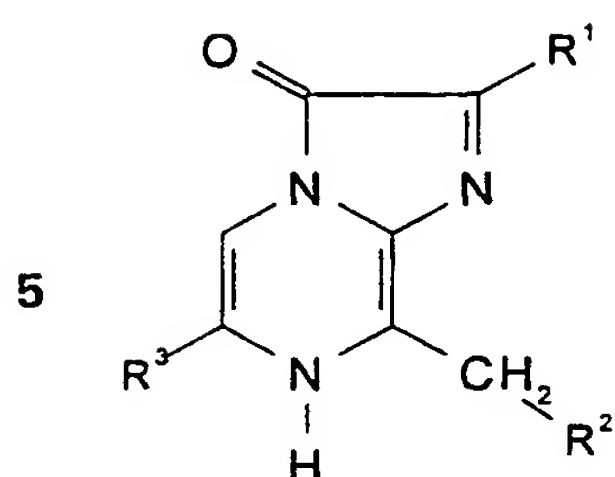
In general for use herein, the components of the bioluminescence are packaged or provided so that there is insufficient metal ions to trigger the reaction. When used, the trace amounts of triggering metal ion, particularly Ca^{2+} is contacted with the other components. For a more sustained glow, aequorin can be continuously reconstituted or can be added or can be provided in high excess.

25 (b) Luciferin

The aequorin luciferin is coelenterazine and analogs therein, which include molecules including the structure [formula (I)]:

30

-61-



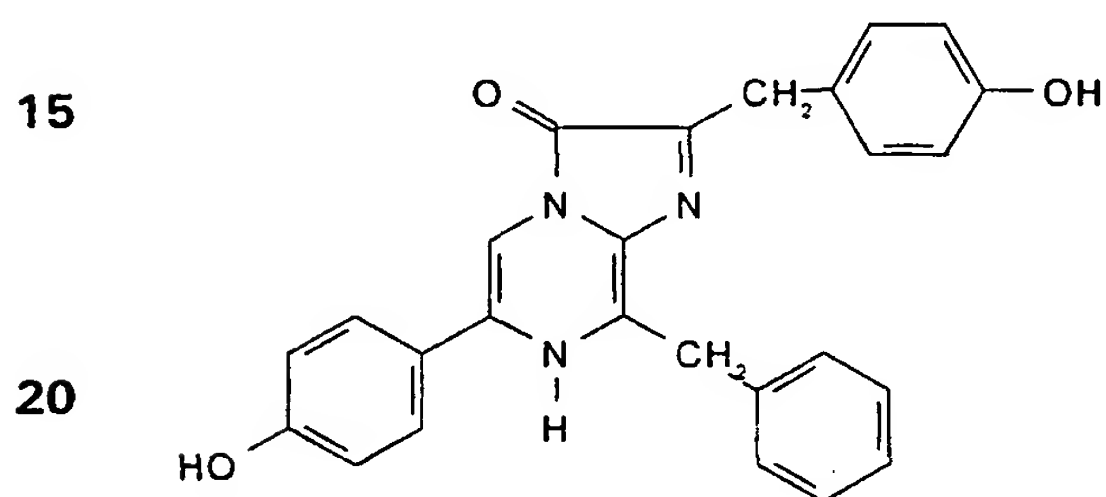
in which R₁ is CH₂C₆H₅ or CH₃; R₂ is C₆H₅, and R₃ is *p*-C₆H₄OH or CH₃ or other such analogs that have activity. Preferred coelenterazine has the structure in which R₁ is *p*-CH₂C₆H₄OH, R₂ is C₆H₅, and R₃ is *p*-C₆H₄OH, which can be prepared by known methods

[see, e.g., Inouye *et al.* (1975) *Jap. Chem. Soc.*,

Chemistry Lttrs. pp 141-144; and Hart *et al.* (1979) *Biochemistry* 18:2204-

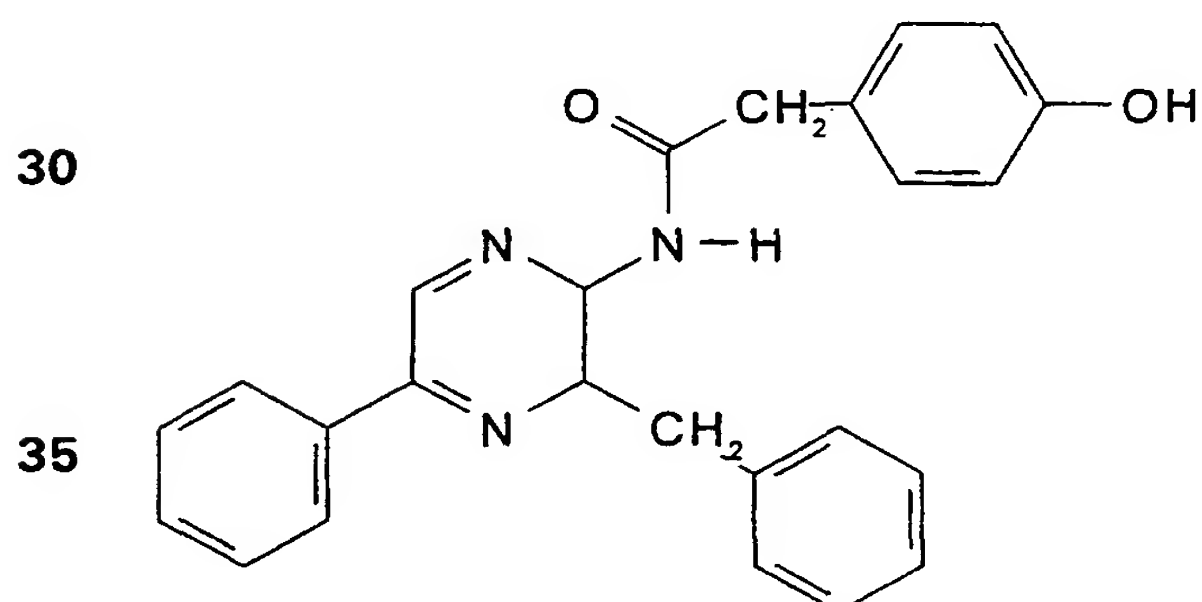
10 2210]. Among the preferred analogs, are those that are modified, whereby the spectral frequency of the resulting light is shifted to another frequency.

The preferred coelenterazine has the structure (II):



and sulfated derivatives thereof.

25 Another coelenterazine has formula (V):



[see, Hart *et al.* (1979)

Biochemistry 18:2204-2210]. Using this derivative in the presence of

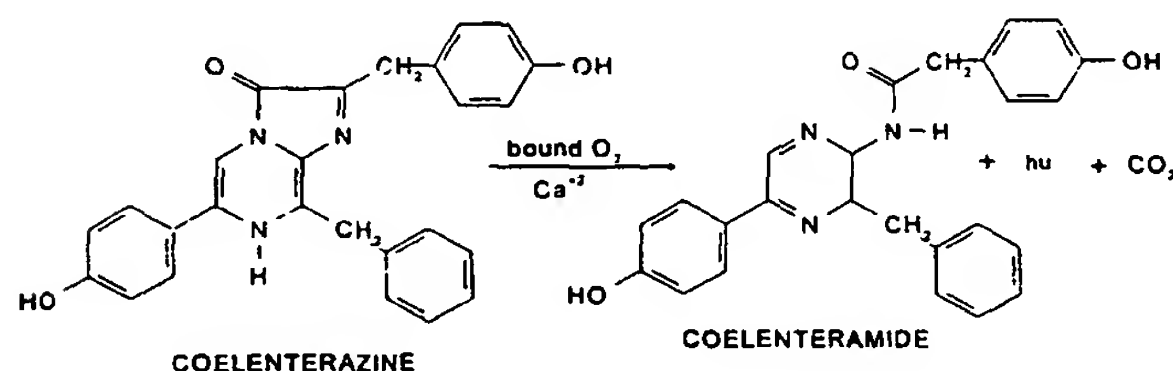
-62-

luciferase all of the light is in the ultraviolet with a peak at 390 nm. Upon addition of GFP, all light emitted is now in the visible range with a peak at 509 nm accompanied by an about 200-fold increase in the amount of light emitted. Viewed with a cut-off filter of 470 nm, in the light yield in the absence of GFP would be about zero, and would be detectable in the presence of GFP. This provides the basis for an immunoassay described in the EXAMPLES.

The reaction of coelenterazine when bound to the aequorin photoprotein with bound oxygen and in the presence of Ca^{2+} can be represented as follows:

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The photoprotein aequorin [which contains apoaequorin bound to a coelenterate luciferin molecule] and *Renilla* luciferase, discussed below, can use the same coelenterate luciferin. The aequorin photoprotein catalyses the oxidation of coelenterate luciferin [coelenterazine] to oxyluciferin [coelenteramide] with the concomitant production of blue light [$\lambda_{\text{max}} = 469 \text{ nm}$].

Importantly, the sulfate derivative of the coelenterate luciferin [lauryl-luciferin] is particularly stable in water, and thus may be used in a coelenterate-like bioluminescent system. In this system, adenosine diphosphate (ADP) and a sulpha-kinase are used to convert the coelenterazine to the sulphated form. Sulfatase is then used to reconvert the lauryl-luciferin to the native coelenterazine. Thus, the more stable lauryl-luciferin is used in the item to be illuminated and the luciferase combined with the sulfatase are added to the luciferin mixture when illumination is desired.

-63-

Thus, the bioluminescent system of *Aequorea* is particularly suitable for use in the methods herein. The particular amounts and the manner in which the components are provided depends upon the type of neoplasia or specialty tissue to be visualized. This system can be provided in lyophilized form, that will glow
5 upon addition of Ca^{2+} . It can be encapsulated, linked to microcarriers, such as microbeads, or in as a compositions, such as a solution or suspension, preferably in the presence of sufficient chelating agent to prevent triggering the reaction. The concentration of the aequorin photoprotein will vary and can be determined empirically. Typically concentrations of at least 0.1 mg/l, more
10 preferably at least 1 mg/l and higher, will be selected. In certain embodiments, 1-10 mg luciferin/100 mg of luciferase will be used in selected volumes and at the desired concentrations will be used.

c. Crustacean, particularly *Cypridina* systems

The ostracods, such as *Vargula serratta*, *hilgendorffii* and *noctiluca* are
15 small marine crustaceans, sometimes called sea fireflies. These sea fireflies are found in the waters off the coast of Japan and emit light by squirting luciferin and luciferase into the water, where the reaction, which produces a bright blue luminous cloud, occurs. The reaction involves only luciferin, luciferase and molecular oxygen, and, thus, is very suitable for application herein.

20 The systems, such as the *Vargula* bioluminescent systems, are particularly preferred herein because the components are stable at room temperature if dried and powdered and will continue to react even if contaminated. Further, the bioluminescent reaction requires only the luciferin/luciferase components in concentrations as low as 1:40 parts per billion
25 to 1:100 parts per billion, water and molecular oxygen to proceed. An exhausted system can renewed by addition of luciferin.

(1) *Vargula* luciferase

The *Vargula* luciferase is water soluble and is among those preferred for use in the methods herein. *Vargula* luciferase is a 555-amino acid polypeptide
30 that has been produced by isolation from *Vargula* and also using recombinant technology by expressing the DNA in suitable bacterial and mammalian host cells [see, e.g., Thompson et al. (1989) Proc. Natl. Acad. Sci. U.S.A. 86:6567-

-64-

6571; Inouye et al. (1992) Proc. Natl. Acad. Sci. U.S.A. 89:9584-9587;
Johnson et al. (1978) Methods in Enzymology LVII:331-349; Tsuji et al. (1978)
Methods Enzymol. 57:364-72; Tsuji (1974) Biochemistry 13:5204-5209;
Japanese Patent Application No. JP 3-30678 Osaka; and European Patent
5 Application No. EP 0 387 355 A1].

(a) **Purification from *Cypridina***

Methods for purification of *Vargula* [*Cypridina*] luciferase are well known.
For example, crude extracts containing the active can be
readily prepared by grinding up or crushing the *Vargula* shrimp. In other
10 embodiments, a preparation of *Cypridina hilgendorfi* luciferase can be prepared
by immersing stored frozen *C. hilgendorfi* in distilled water containing, 0.5-5.0
M salt, preferably 0.5-2.0 M sodium or potassium chloride, ammonium sulfate,
at 0-30° C, preferably 0-10° C, for 1-48 hr, preferably 10-24 hr, for extraction
followed by hydrophobic chromatography and then ion exchange or affinity
15 chromatography [TORAY IND INC, Japanese patent application JP 4258288,
published September 14, 1993; see, also, Tsuji et al. (1978) Methods Enzymol.
57:364-72 for other methods].

(b) **Preparation by Recombinant Methods**

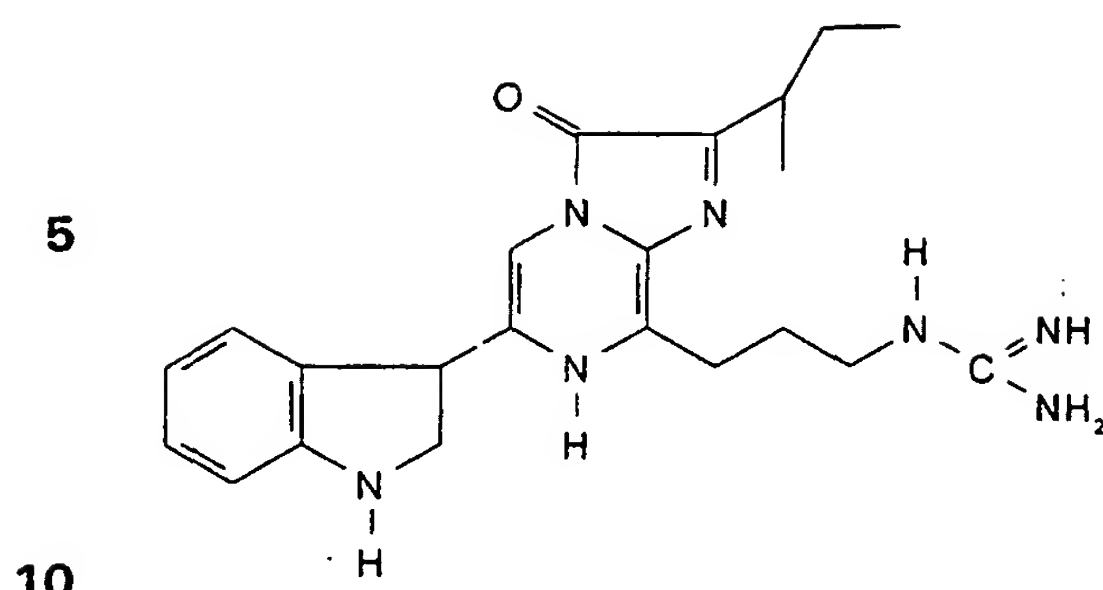
The luciferase is preferably produced by expression of cloned DNA
20 encoding the luciferase [European Patent Application No. 0 387 355 A1;
International PCT Application No. WO 95/001542; see, also SEQ ID No. 5,
which sets forth the sequence from Japanese Patent Application No. JP 3-
30678 and Thompson et al. (1989) Proc. Natl. Acad. Sci. U.S.A. 86:6567-
6571] DNA encoding the luciferase or variants thereof is introduced into *E. coli*
25 using appropriate vectors and isolated using standard methods.

(2) ***Vargula* luciferin**

The natural luciferin is a substituted imidazopyrazine nucleus, such a
compound of formula (III):

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-65-



The luciferin can be isolated from ground dried *Vargula* by heating the extract, which destroys the luciferase but leaves the

luciferin intact [see, e.g., U.S. Patent No. 4,853,327].

Analogous thereof and other compounds that react with the luciferase in a light producing reaction also may be used.

15 Other bioluminescent organisms that have luciferases that can react with the *Vargula* luciferin include, the genera *Apogon*, *Parapriacanthus* and *Porichthys*.

(3) Reaction

20 The luciferin upon reaction with oxygen forms a dioxetanone intermediate [which includes a cyclic peroxide similar to the firefly cyclic peroxide molecule intermediate]. In the final step of the bioluminescent reaction, the peroxide breaks down to form CO₂ and an excited carbonyl. The excited molecule then emits a blue to blue-green light.

25 The optimum pH for the reaction is about 7. For purposes herein, any pH at which the reaction occurs may be used. The concentrations of reagents are those normally used for analytical reactions or higher [see, e.g., Thompson et al. (1990) Gene 96:257-262]. Typically concentrations of the luciferase between 0.1 and 10 mg/l, preferably 0.5 to 2.5 mg/l will be used. Similar concentrations or higher concentrations of the luciferin may be used.

30 d. Insect bioluminescent systems including fireflies, click beetles, and other insect system

The biochemistry of firefly bioluminescence was the first bioluminescent system to be characterized [see, e.g.,

-66-

Wienhausen et al. (1985) Photochemistry and Photobiology 42:609-611;
McElroy et al. (1966) in *Molecular Architecture in cell Physiology*, Hayashi et al., eds. Prentice Hall, Inc., Englewood Cliffs, NJ, pp. 63-80] and it is
commercially available [e.g., from Promega Corporation, Madison, WI, see, e.g.,
5 Leach et al. (1986) Methods in Enzymology 133:51-70, esp. Table 1].
Luciferases from different species of fireflies are antigenically similar. These
species include members of the genera *Photinus*, *Photurins* and *Luciola*.
Further, the bioluminescent reaction produces more light at 30°C than at 20°C,
the luciferase is stabilized by small quantities of bovine albumin serum, and the
10 reaction can be buffered by tricine.

(1) Luciferase

DNA clones encoding luciferases from various insects and the use to
produce the encoded luciferase is well known. For example, DNA clones that
encode luciferase from *Photinus pyralis*, *Luciola cruciata* [see, e.g., de Wet et al.
15 (1985) Proc. Natl. Acad. Sci. U.S.A. 82:7870-7873; de We et al. (1986)
Methods in Enzymology 133:3; U.S. Patent No. 4,968,613, see, also SEQ ID
No. 3] are available. The DNA has also been expressed in *Saccharomyces* [see,
e.g., Japanese Application No. JP 63317079, published December 26, 1988,
KIKKOMAN CORP] and in tobacco.

20 In addition to the wild-type luciferase modified insect luciferases have
been prepared. For example, heat stable luciferase mutants, DNA-encoding the
mutants, vectors and transformed cells for producing the luciferases are
available. A protein with 60% amino acid sequence homology with luciferases
from *Photinus pyralis*, *Luciola mingrelica*, *L. cruciata* or *L. lateralis* and having
25 luciferase activity is available [see, e.g., International PCT Application No. WO
95/25798]. It is more stable above 30° C than naturally-occurring insect
luciferases and may also be produced at 37° C or above, with higher yield.

Modified luciferases that generate light at different wavelengths
[compared with native luciferase], and thus, may be selected for their color-
30 producing characteristics. For example, synthetic mutant beetle luciferase(s)
and DNA encoding such luciferases that produce bioluminescence at a
wavelength different from wild-type luciferase are known [Promega Corp,

-67-

International PCT Application No. WO 95/18853, which is based on U.S. application Serial No. 08/177,081]. The mutant beetle luciferase has an amino acid sequence differing from that of the corresponding wild-type *Luciola cruciata* [see, e.g., U.S. Patent Nos. 5,182,202, 5,219,737, 5,352,598, see, also SEQ
5 ID No.3] by a substitution(s) at one or two positions. The mutant luciferase produces a bioluminescence with a wavelength of peak intensity that differs by at least 1 nm from that produced by wild-type luciferases.

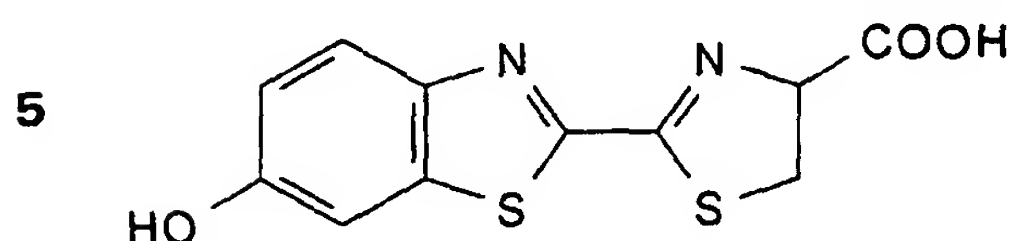
Other mutant luciferases can be produced. Mutant luciferases with the amino acid sequence of wild-type luciferase, but with at least one mutation in
10 which valine is replaced by isoleucine at the amino acid number 233, valine by isoleucine at 239, serine by asparagine at 286, glycine by serine at 326, histidine by tyrosine at 433 or proline by serine at 452 are known [see, e.g., U.S. Patent Nos. 5,219,737, and 5,330,906]. The luciferases are produced by expressing DNA-encoding each mutant luciferase in E. coli and isolating the
15 protein. These luciferases produce light with colors that differ from wild-type. The mutant luciferases catalyze luciferin to produce red [λ 609 nm and 612 nm], orange [λ 595 and 607 nm] or green [λ 558 nm] light. The other physical and chemical properties of mutant luciferase are substantially identical to native wild type-luciferase. The mutant luciferase has the amino acid sequence of
20 *Luciola cruciata* luciferase with an alteration selected from Ser 286 replaced by Asn, Gly 326 replaced by Ser, His 433 replaced by Tyr or Pro 452 replaced by Ser. Thermostable luciferases are also available [see, e.g., U.S. Patent No. 5,229,285; see, also International PCT Application No. WO 95/25798, which provides *Photinus* luciferase in which the glutamate at position 354 is replaced
25 lysine and *Luciola* luciferase in which the glutamate at 356 is replaced with lysine].

These mutant luciferases as well as the wild type luciferases can be used in combination with the GFPs provided herein particularly in instances when a variety of colors are desired or when stability at higher temperatures is desired.

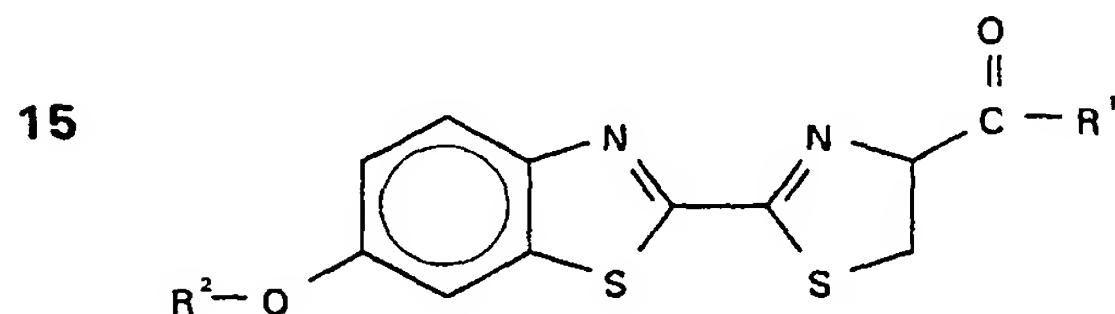
-68-

(2) Luciferin

The firefly luciferin is a benzothiazole:



10 Analogs of this luciferin and synthetic firefly luciferins are also known to those of skill in art [see, e.g., U.S. Patent No. 5,374,534 and 5,098,828]. These include compounds of formula (IV) [see, U.S. Patent No. 5,098,828]:



20 in which:

R¹ is hydroxy, amino, linear or branched C₁-C₂₀ alkoxy, C₂-C₂₀ alkenyloxy, an L-amino acid radical bond via the α-amino group, an oligopeptide radical with up to ten L-amino acid units linked via the α-amino group of the terminal unit;

25 R² is hydrogen, H₂PO₃, HSO₃, unsubstituted or phenyl substituted linear or branched C₁-C₂₀ alkyl or C₂-C₂₀ alkenyl, aryl containing 6 to 18 carbon atoms, or R³-C(O)-; and

30 R³ is an unsubstituted or phenyl substituted linear or branched C₁-C₂₀ alkyl or C₂-C₂₀ alkenyl, aryl containing 6 to 18 carbon atoms, a nucleotide radical with 1 to 3 phosphate groups, or a glycosidically attached mono- or disaccharide, except when formula (IV) is a D-luciferin or D-luciferin methyl ester.

Modified luciferins that have been modified to produce light of shifted frequencies are known to those of skill in the art.

35 (3) Reaction

The reaction catalyzed by firefly luciferases and related insect luciferases requires ATP, Mg²⁺ as well as molecular oxygen. Luciferin must be added

-69-

exogenously. Firefly luciferase catalyzes the firefly luciferin activation and the subsequent steps leading to the excited product. The luciferin reacts with ATP to form a luciferyl adenylate intermediate. This intermediate then reacts with oxygen to form a cyclic luciferyl peroxy species, similar to that of the
5 coelenterate intermediate cyclic peroxide, which breaks down to yield CO₂ and an excited state of the carbonyl product. The excited molecule then emits a yellow light; the color, however, is a function of pH. As the pH is lowered the color of the bioluminescence changes from yellow-green to red.

Different species of fireflies emit different colors of bioluminescence so
10 that the color of the reaction will be dependent upon the species from which the luciferase is obtained. Additionally, the reaction is optimized at pH 7.8.

Addition of ATP and luciferin to a reaction that is exhausted produces additional light emission. Thus, the system, once established, is relatively easily maintained. Therefore, it is highly suitable for use herein in embodiments in
15 which a sustained glow is desired.

e. Bacterial systems

Luminous bacteria typically emit a continuous light, usually blue-green. When strongly expressed, a single bacterium may emit 10⁴ to 10⁵ photons per second. Bacterial bioluminescence systems include, among others, those
20 systems found in the bioluminescent species of the genera *Photobacterium*, *Vibrio* and *Xenorhabdus*. These systems are well known and well characterized [see, e.g., Baldwin *et al.* (1984) *Biochemistry* 23:3663-3667; Nicoli *et al.* (1974) *J. Biol. Chem.* 249:2393-2396; Welches *et al.* (1981) *Biochemistry* 20:512-517; Engebrecht *et al.* (1986) *Methods in Enzymology* 133:83-99;
25 Frackman *et al.* (1990) *J. of Bacteriology* 172:5767-5773; Miyamoto *et al.* (1986) *Methods in Enzymology* 133:70; U.S. Patent No. 4,581,335].

(1) Luciferases

Bacterial luciferase, as exemplified by luciferase derived from *Vibrio harveyi* [EC 1.14.14.3, alkanol reduced-FMN-oxygen oxidoreductase 1-hydroxylating,
30 luminescing], is a mixed function oxidase, formed by the association of two different protein subunits α and β . The α -subunit has an apparent molecular weight of approximately 42,000 kD and the β -subunit has an apparent

-70-

molecular weight of approximately 37,000 kD [see, e.g., Cohn et al. (1989) Proc. Natl. Acad. Sci. U.S.A. 90:102-123]. These subunits associate to form a 2-chain complex luciferase enzyme, which catalyzes the light emitting reaction of bioluminescent bacteria, such as *Vibrio harveyi* [U.S. Patent No. 4,581,335; 5 Belas et al. (1982) Science 218:791-793], *Vibrio fischeri* [Engebrecht et al. (1983) Cell 32:773-781; Engebrecht et al. (1984) Proc. Natl. Acad. Sci. U.S.A. 81:4154-4158] and other marine bacteria.

Bacterial luciferase genes have been cloned [see, e.g., U.S. Patent No. 5,221,623; U.S. Patent No. 4,581,335; European Patent Application No. EP 10 386 691 A]. Plasmids for expression of bacterial luciferase, such as *Vibrio harveyi*, include pFIT001 (NRRL B-18080), pPALE001 (NRRL B-18082) and pMR19 (NRRL B-18081)] are known. For example the sequence of the entire *lux* regulon from *Vibrio fischeri* has been determined [Baldwin et al. (1984), Biochemistry 23:3663-3667; Baldwin et al. (1981) Biochem. 20: 512-517; 15 Baldwin et al. (1984) Biochem. 23:3663-3667; see, also, e.g., U.S. Patent Nos. 5,196,318, 5,221,623, and 4,581,335]. This regulon includes *luxI* gene, which encodes a protein required for autoinducer synthesis [see, e.g., Engebrecht et al. (1984) Proc. Natl. Acad. Sci. U.S.A. 81:4154-4158], the *luxC*, *luxD*, and *luxE* genes, which encode enzymes that provide the luciferase 20 with an aldehyde substrate, and the *luxA* and *luxB* genes, which encode the alpha and beta subunits of the luciferase.

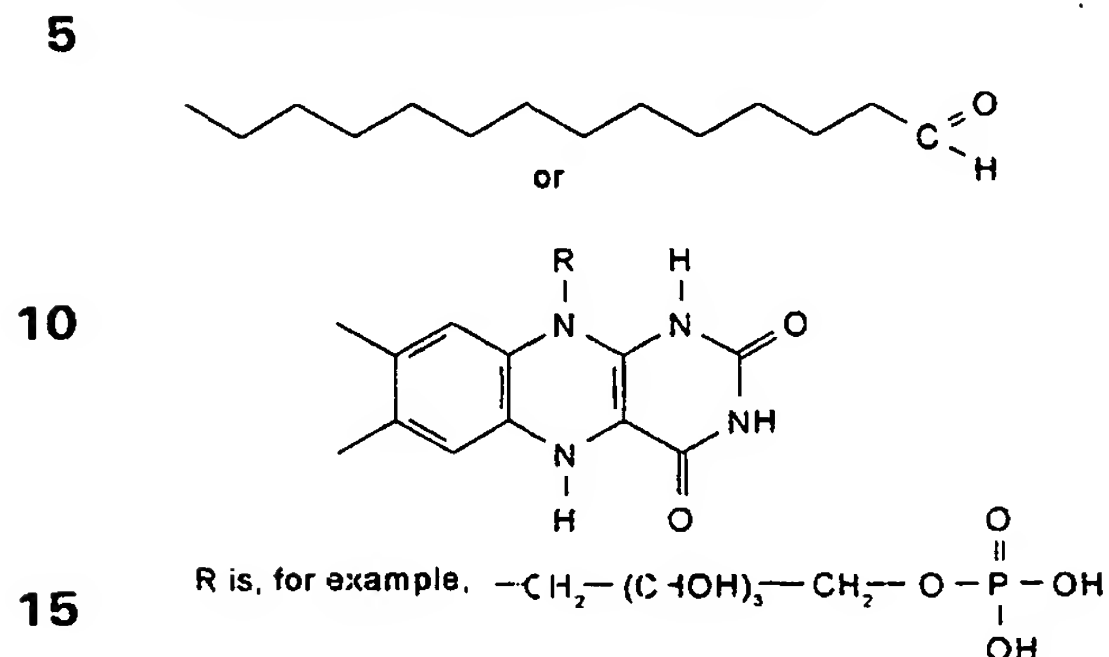
Lux genes from other bacteria have also been cloned and are available [see, e.g., Cohn et al. (1985) J. Biol. Chem. 260:6139-6146; U.S. Patent No. 5,196,524, which provides a fusion of the *luxA* and *luxB* genes from *Vibrio* 25 *harveyi*]. Thus, luciferase alpha and beta subunit-encoding DNA is provided and can be used to produce the luciferase. DNA encoding the α [1065 bp] and β [984 bp] subunits, DNA encoding a luciferase gene of 2124 bp, encoding the alpha and beta subunits, a recombinant vector containing DNA encoding both subunits and a transformed *E. coli* and other bacterial hosts for expression and

-71-

production of the encoded luciferase are available. In addition, bacterial luciferases are commercially available.

(2) Luciferins

Bacterial luciferins include:



in which the tetradecanal with reduced flavin mononucleotide are considered luciferin since both are oxidized during the light emitting reaction.

(3) Reactions

The bacterial systems require, in addition to reduced flavin, five polypeptides to complete the bioluminescent reaction: two subunits, α and β , of bacterial luciferin and three units of a fatty acid reductase system complex, which supplies the tetradecanal aldehyde. Examples of bacterial bioluminescent systems useful in the apparatus and methods provided herein include those derived from *Vibrio fischeri* and *Vibrio harveyi*. One advantage to this system is its ability to operate at cold temperatures; certain surgical procedures are performed by cooling the body to lower temperatures.

30 Bacterial luciferase catalyzes the flavin-mediated hydroxylation of a long-chain aldehyde to yield carboxylic acid and an excited flavin; the flavin decays to ground state with the concomitant emission of blue green light [$\lambda_{\text{max}} = 490 \text{ nm}$; see, e.g., Legocki et al. (1986) Proc. Natl. Acad. Sci. USA 81:9080; see U.S. Patent No. 5,196,524]:

-72-



The reaction can be initiated by contacting reduced flavin mononucleotide [FMNH₂] with a mixture of the bacterial luciferase, oxygen, and a long-chain aldehyde, usually n-decyl aldehyde.

DNA encoding luciferase from the fluorescent bacterium *Alteromonas*
 5 *hanedai* is known [CHISSO CORP; see, also, Japanese application JP 7222590, published August 22, 1995]. The reduced flavin mononucleotide [FMNH₂; luciferin] reacts with oxygen in the presence of bacterial luciferase to produce an intermediate peroxy flavin. This intermediate reacts with a long-chain aldehyde [tetradecanal] to form the acid and the luciferase-bound hydroxy flavin
 10 in its excited state. The excited luciferase-bound hydroxy flavin then emits light and dissociates from the luciferase as the oxidized flavin mononucleotide [FMN] and water. In vivo FMN is reduced again and recycled, and the aldehyde is regenerated from the acid.

Flavin reductases have been cloned [see, e.g., U.S. Patent No.
 15 5,484,723; see, SEQ ID No. 14 for a representative sequence from this patent]. These as well as NAD(P)H can be included in the reaction to regenerate FMNH₂ for reaction with the bacterial luciferase and long chain aldehyde. The flavin reductase catalyzes the reaction of FMN, which is the luciferase reaction, into FMNH₂; thus, if luciferase and the reductase are included in the reaction
 20 system, it is possible to maintain the bioluminescent reaction. Namely, since the bacterial luciferase turns over many times, bioluminescence continues as long as a long chain aldehyde is present in the reaction system.

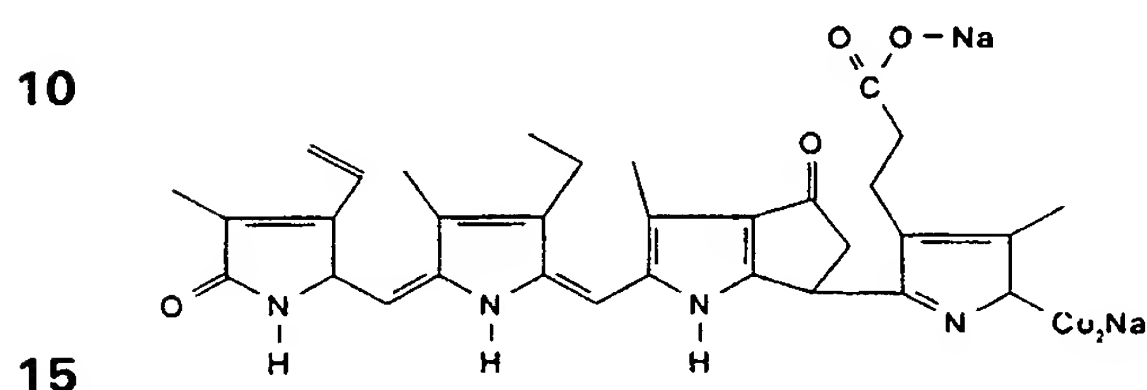
The color of light produced by bioluminescent bacteria also results from the participation of a protein blue-fluorescent protein [BFP] in the
 25 bioluminescence reaction. This protein, which is well known [see, e.g., Lee et al. (1978) Methods in Enzymology LVII:226-234], may also be added to bacterial bioluminescence reactions in order to cause a shift in the color.

-73-

f. Other systems

(1) Dinoflagellate bioluminescence generating systems

In dinoflagellates, bioluminescence occurs in organelles termed scintillons. These organelles are outpocketings of the cytoplasm into the cell vacuole. The scintillons contain only dinoflagellate luciferase and luciferin [with its binding protein], other cytoplasmic components being somehow excluded. The dinoflagellate luciferin is a tetrapyrrole related to chlorophyll:



or an analog thereof.

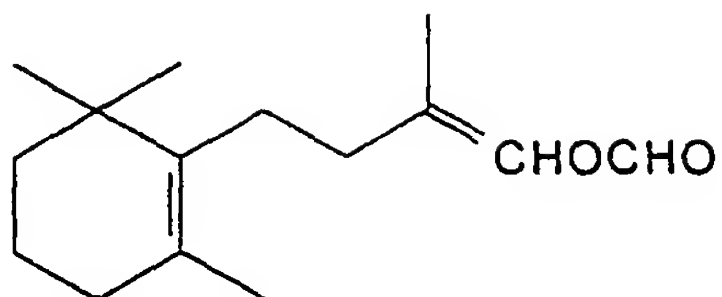
The luciferase is a 135 kD single chain protein that is active at pH 6.5, but inactive at pH 8 [see, e.g., Hastings (1981) Bioluminescence and Chemiluminescence, DeLuca et al., eds. Academic Press, NY, pp.343-360]. Luminescent activity can be obtained in extracts made at pH 8 by simply shifting the pH from 8 to 6. This occurs in soluble and particulate fractions. Within the intact scintillon, the luminescent flash occurs for ~100 msec, which is the duration of the flash *in vivo*. In solution, the kinetics are dependent on dilution, as in any enzymatic reaction. At pH 8, the luciferin is bound to a protein [luciferin binding protein] that prevents reaction of the luciferin with the luciferase. At pH 6, however, the luciferin is released and free to react with the enzyme.

(2) Systems from molluscs, such as *Latia* and *Pholas*

Molluscs *Latia neritoides* and species of *Pholas* are bioluminescent animals. The luciferin has the structure:

35

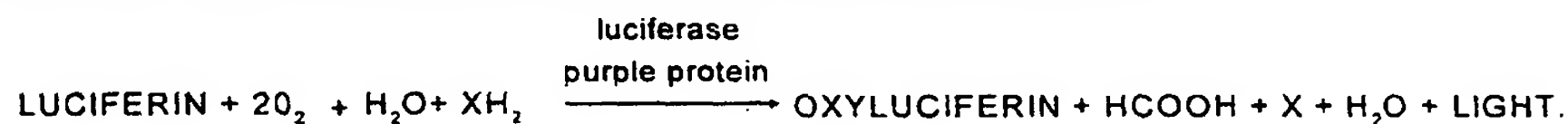
-74-



and has been synthesized [see, e.g., Shimomura et al. (1968) Biochemistry 7:1734-1738; Shimomura et al. (1972) Proc. Natl. Acad. Sci. U.S.A. 69:2086-2089].

5

In addition to a luciferase and luciferin the reaction has a third component, a "purple protein". The reaction, which can be initiated by an exogenous reducing agent is represented by the following scheme:



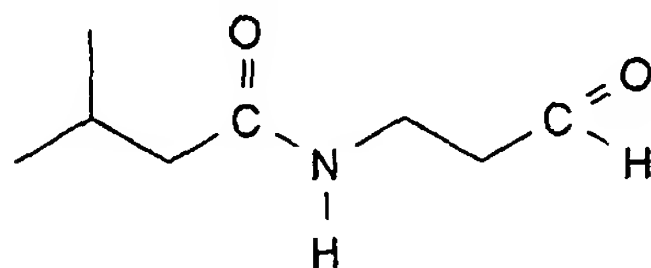
XH_2 is a reducing agent.

10 Thus for practice herein, the reaction will require the purple protein as well as a reducing agent.

(3) Earthworms and other annelids

Earthworm species, such as *Diplocardia longa*, *Chaetopterus* and *Harmothoe*, exhibit bioluminescence. The luciferin has the structure:

15



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The reaction requires hydrogen peroxide in addition to luciferin and luciferase. The luciferase is a photoprotein.

(4) Glow worms

30 The luciferase/luciferin system from the glow worms that are found in Great Britain, and in Australian and New Zealand caves are also intended for use herein.

-75-

(5) Marine polychaete worm systems

Marine polychaete worm bioluminescence generating systems, such as *Phyxotrix* and *Chaetopterus*, are also contemplated for use herein.

(6) South American railway beetle

- 5 The bioluminescence generating system from the South American railway beetle is also intended for use herein.

(7) Fish

- Of interest herein, are luciferases and bioluminescence generating systems that generate red light. These include luciferases found in species of
10 *Aristostomias*, such as *A. scintillans* [see, e.g., O'Day et al. (1974) Vision Res. 14:545-550], *Pachystomias*, *Malacosteus*, such as *M. niger*.

Blue/green emitters include cyclothone, myctophids, hatchet fish (agyropelecus), vinciguerria, howella, florenciella, and Chauliodus.

g. Fluorescent Proteins

- 15 The GFP from *Aequorea* and that of the sea pansy *Renilla reniformis* share the same chromophore, yet *Aequorea* GFP has two absorbance peaks at 395 and 475 nm, whereas *Renilla* GFP has only a single absorbance peak at 498 nm, with about 5.5 fold greater monomer extinction coefficient than the major 395 nm peak of the *Aequorea* protein [Ward, W. W. in Bioluminescence and Chemiluminescence (eds. DeLuca, M. A. & McElroy, W. D.) 235-242
20 (Academic Press, New York, 1981)]. The spectra of the isolated chromophore and denatured protein at neutral pH do not match the spectra of either native protein [Cody, C. W. et al. (1993) Biochemistry 32:1212-1218].

(1) Green and blue fluorescent proteins

- 25 As described herein, blue light is produced using the *Renilla* luciferase or the *Aequorea* photoprotein in the presence of Ca^{2+} and the coelenterazine luciferin or analog thereof. This light can be converted into a green light if a green fluorescent protein (GFP) is added to the reaction. Green fluorescent proteins, which have been purified [see, e.g., Prasher et al. (1992) Gene
30 111:229-233] and also cloned [see, e.g., International PCT Application No. WO 95/07463, which is based on U.S. application Serial No. 08/119,678 and U.S. application Serial No. 08/192,274, which are herein incorporated by reference],

-76-

are used by cnidarians as energy-transfer acceptors. GFPs fluoresce in vivo upon receiving energy from a luciferase-oxyluciferin excited-state complex or a Ca^{2+} -activated photoprotein. The chromophore is modified amino acid residues within the polypeptide. The best characterized GFPs are those of *Aequorea* and
5 *Renilla* [see, e.g., Prasher et al. (1992) Gene 111:229-233; Hart, et al. (1979) Biochemistry 18:2204-2210]. For example, a green fluorescent protein [GFP] from *Aequorea victoria* contains 238 amino acids, absorbs blue light and emits green light. Thus, inclusion of this protein in a composition containing the aequorin photoprotein charged with coelenterazine and oxygen, can, in the
10 presence of calcium, result in the production of green light. Thus, it is contemplated that GFPs may be included in the bioluminescence generating reactions that employ the aequorin or *Renilla* luciferases or other suitable luciferase in order to enhance or alter color of the resulting bioluminescence.

GFPs are activated by blue light to emit green light and thus may be used
15 in the absence of luciferase and in conjunction with an external light source with novelty items, as described herein. Similarly, blue fluorescent proteins (BFPs), such as from *Vibrio fischeri*, *Vibrio harveyi* or *Photobacterium phosphoreum*, may be used in conjunction with an external light source of appropriate wavelength to generate blue light. (See for example, Karatani, et al., "A blue fluorescent protein from a yellow-emitting luminous bacterium,"
20 Photochem. Photobiol. 55(2):293-299 (1992); Lee, et al., "Purification of a blue-fluorescent protein from the bioluminescent bacterium *Photobacterium phosphoreum*" Methods Enzymol. (Biolumin. Chemilumin.) 57:226-234 (1978); and Gast, et al. "Separation of a blue fluorescence protein from bacterial
25 luciferase" Biochem. Biophys. Res. Commun. 80(1):14-21 (1978), each, as all references cited herein, incorporated in its entirety by reference herein.) In particular, GFPs, and/or BFPs or other such fluorescent proteins may be used in the beverage and/or food combinations provided herein and served in rooms illuminated with light of an appropriate wavelength to cause the fluorescent
30 proteins to fluoresce.

GFPs and/or BFPs or other such fluorescent proteins may be used in any of the novelty items and combinations provided herein, such as the beverages

-77-

and toys, including bubble making toys, particularly bubble-making compositions or mixtures. Also of particular interest are the use of these proteins in cosmetics, particularly face paints or make-up, hair colorants or hair conditioners, mousses or other such products. Such systems are particularly of interest because no luciferase is needed to activate the photoprotein and because the proteins are non-toxic and safe to apply to the skin, hair, eyes and to ingest. These fluorescent proteins may also be used in addition to bioluminescence generating systems to enhance or create an array of different colors.

These proteins may be used alone or in combination with bioluminescence generating systems to produce an array of colors. They may be used in combinations such that the color of, for example, a beverage changes over time, or includes layers of different colors.

(2) Phycobiliproteins

Phycobiliproteins are water soluble fluorescent proteins derived from cyanobacteria and eukaryotic algae [see, e.g., Apt *et al.* (1995) *J. Mol. Biol.* 238:79-96; Glazer (1982) *Ann. Rev. Microbiol.* 36:173-198; and Fairchild *et al.* (1994) *J. of Biol. Chem.* 269:8686-8694]. These proteins have been used as fluorescent labels in immunoassay [see, Kronick (1986) *J. of Immunolog. Meth.* 92:1-13], the proteins have been isolated and DNA encoding them is also available [see, e.g., Pilot *et al.* (1984) *Proc. Natl. Acad. Sci. U.S.A.* 81:6983-6987; Lui *et al.* (1993) *Plant Physiol* 103:293-294; and Houmard *et al.* (1988) *J. Bacteriol.* 170:5512-5521; the proteins are commercially available from, for example, ProZyme, Inc., San Leandro, CA].

In these organisms, the phycobiliproteins are arranged in subcellular structures termed phycobilisomes, and function as accessory pigments that participate in photosynthetic reactions by absorbing visible light and transferring the derived energy to chlorophyll via a direct fluorescence energy transfer mechanism.

Two classes of phycobiliproteins are known based on their color: phycoerythrins (red) and phycocyanins (blue), which have reported absorbtion maxima between 490 and 570 nm and between 610 and 665 nm, respectively.

-78-

Phycoerythrins and phycocyanins are heterogenous complexes composed of different ratios of alpha and beta monomers to which one or more class of linear tetrapyrrole chromophores are covalently bound. Particular phycobiliproteins may also contain a third γ -subunit which often associated with $(\alpha\beta)_6$ aggregate proteins.

All phycobiliproteins contain either phycothrombilin or phycoerythrobilin chromophores, and may also contain other bilins phycourobilin, cryptoviolin or the 697 nm bilin. The γ -subunit is covalently bound with phycourobilin which results in the 495-500 nm absorption peak of B- and R-phycoerythrins. Thus, the spectral characteristics of phycobiliproteins may be influenced by the combination of the different chromophores, the subunit composition of the apo-phycobiliproteins and/or the local environment effecting the tertiary and quaternary structure of the phycobiliproteins.

As described above for GFPs and BFPs, phycobiliproteins are also activated by visible light of the appropriate wavelength and, thus, may be used in the absence of luciferase and in conjunction with an external light source to illuminate neoplasia and specialty tissues, as described herein. Furthermore, the attachment of phycobiliproteins to solid support matrices is known (e.g., see U.S. Patent Nos. 4,714,682; 4,767,206; 4,774,189 and 4,867,908). As noted above, these proteins may be used in combination with other fluorescent proteins and/or bioluminescence generating systems to produce an array of colors or to provide different colors over time.

As described above, attachment of phycobiliproteins to solid support matrices is known (e.g., see U.S. Patent Nos. 4,714,682; 4,767,206; 4,774,189 and 4,867,908). Therefore, phycobiliproteins may be coupled to microcarriers coupled to one or more components of the bioluminescent reaction, preferably a luciferase, to convert the wavelength of the light generated from the bioluminescent reaction. Microcarriers coupled to one or more phycobiliproteins may be used in any of the methods provided herein.

The conversion of blue or green light to light of a longer wavelength, i.e., red or near infra-red, is particularly preferred for the visualization of deep

-79-

neoplasias or specialty tissues using a laparoscope or computer tomogram imaging system, as described herein.

Thus, when a change in the frequency of emitted light is desired, the phycobiliprotein can be included with the bioluminescent generating

5 components.

C. ISOLATION AND IDENTIFICATION OF NUCLEIC ACIDS ENCODING LUCIFERASES AND GFPs

Nucleic acid bioluminescent proteins, including two new green fluorescent proteins (GFPs) and three coelenterazine-using luciferases are
10 provided. An advantage of the coelenterazine-using luciferases in many applications, particularly analytical applications, is that only the light-emitting luciferin and molecular oxygen are needed; cofactors such as ATP or Ca^{++} are not required.

The nucleic acids that encode these luciferases and GFPs can also be
15 used to isolated related nucleic acid from related speices. Also provided herein, are methods for isolating additional genes encoding luciferases and, particularly GFPs, from related species that have heretofore proven difficult to isolate.

Nucleic acids encoding luciferases from *Renilla mulleri*, *Pleuromamma*, *Gaussia* and *Ptilosarcus* have been isolated. These nucleic acids have been or
20 can be introduced into plasmids and expression vectors and into suitable host cells. The host cells have been and can be used to produce the encoded protein, which can be used for any of the applications described herein or known to those of skill in the art.

The cloned DNA fragments can be replicated in bacterial cells, preferably
25 in *E. coli*. A preferred DNA fragment also includes a bacterial origin of replication, to ensure the maintenance of the DNA fragment from generation to generation of the bacteria. In this way, large quantities of the DNA fragment can be produced by replication in bacteria. Preferred bacterial origins of replication include, but are not limited to, the f1-ori and col E1 origins of
30 replication. Preferred hosts contain chromosomal copies of DNA encoding T7 RNA polymerase operably linked to an inducible promoter, such as the lacUV promoter (see, U.S. Patent No. 4,952,496). Such hosts include, but are not

-80-

limited to, lysogens *E. coli* strains HMS174(DE3)pLysS, BL21(DE3)pLysS, HMS174(DE3) and BL21(DE3). Strain BL21(DE3) is preferred. The pLys strains provide low levels of T7 lysozyme, a natural inhibitor of T7 RNA polymerase.

- 5 For expression and for preparation of mutueins, such as temperature sensitive muteins, eukaryotic cells, among them, yeast cells, such as *Saccharomyces* are preferred.

Fusion proteins of the luciferases and GFPs are also provided. Methods of use thereof are also provided.

- 10 The methods are described with respect to *Renilla* and *Gaussia* nucleic acids and proteins. Similar methods were used to identify and isolated the *Ptilosarcus* and *Pleuromamma* nucleic acids and proteins provided herein.

- The GFP cloned from *Renilla mullerei* has spectral properties that make it extremely useful. These properties include very high quantum efficiency, high
15 molar absorbency and efficient use with universally available fluorescein filters (e.g., Endo GFP filter set sold by Chroma). It is known that *Renilla reniformis* GFP is sixfold brighter than the wild-type *Aequorea* GFP on a molar basis, and three to fourfold brighter than the brightest mutant. The *Renilla mulerei* GFP encoded by the nucleic acid clones provided herein exhibits similar functional
20 characteristics, and the spectra appear identical with those from native *reniformis* GFP.

Based on the excitation and emission curve shapes, the *Ptilosarcus* GFP provided herein has a molar absorbance even higher than that of the *R. mullerei* GFP and should be even brighter.

- 25 The *Guassia* and *Pleuromamma* luciferases are the first two copepod luciferases to be cloned; both are excreted, and so should effective markers for secreted proteins. The *Guassia* luciferase is the smallest luciferase so far found (MW 19,900). All the luciferases show the typical output spectrum of
30 coelenterazine-using luciferases. All show a strong dependance on cation concentration, but do not require divalent cations (data not shown). None of the luciferases has any significant homology with the luciferases isolated from another species.

-81-

There, however, is considerable homology between the *Ptilosarcus* GFP and the *R. mulleri* GFP (~80%) but little homology with the *A. victoria* GFP (~25%). In spite of this, all three proteins are 238AA in length, suggesting that the structures of all three proteins are similar. Sequence comparison
5 among the GFPs isolated from *Aequorea victoria*, *Renilla mullerei*, and *Ptilosarcus* reveal that the chromophore sequences of *R. mullerei* and *Ptilosarcus* are identical, and differ from *A. victoria*. These sequence differences point to protein sites that can be modified without affecting the essential fluorescence properties and also provide a means to identify residues
10 that change these properties.

ISOLATION AND IDENTIFICATION OF NUCLEIC ACID ENCODING *Gaussia* LUCIFERASE

1. Isolation of specimens of the genus *Gaussia*

Specimens of *Gaussia* are readily available from the oceans of the world,
15 including the Gulf of Mexico, Pacific Ocean and Atlantic Ocean. The species used herein for isolation of the exemplified nucleic acid were isolated from the Pacific Ocean off of the Southern California coast in the San Pedro and San Clemente basins. The creatures are identified by sifting through samples of ocean water in the dark and selecting the glowing copepods. Upon capture, the
20 specimens are washed thoroughly and may also be dissected to enrich for light-emitting tissues. The whole organisms or dissected tissues are then snap frozen and stored in liquid nitrogen.

As described in detail in the examples below, whole *Gaussia* were used as a source for isolation of nucleic acids encoding *Gaussia* luciferase (e.g., see
25 SEQ ID No. 19.

2. Preparation of *Gaussia* cDNA expression libraries

Gaussia cDNA expression libraries may be prepared from intact RNA following the methods described herein or by other methods known to those of skill in the art (e.g., see Sambrook et al. (1989) Molecular Cloning: A Laboratory
30 Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.; U.S. Patent No. 5,292,658).

-82-

Typically, the preparation of cDNA libraries includes the isolation of polyadenylated RNA from the selected organism followed by single-strand DNA synthesis using reverse transcriptase, digestion of the RNA strand of the DNA/RNA hybrid and subsequent conversion of the single-stranded DNA to
5 double stranded cDNA.

a. RNA isolation and cDNA synthesis

Whole *Gaussia* was used as source of total cytoplasmic RNA for the preparation of *Gaussia* cDNA. Total intact RNA can be isolated using standard techniques well known to those of skill in the art (e.g., see Sambrook *et al.*
10 (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). After isolating total cellular RNA, polyadenylated RNA species are then easily separated from the nonpolyadenylated species using affinity chromatography on oligodeoxythymidylate cellulose columns, (e.g., as described by Aviv *et al.*,
15 (1972) Proc. Natl. Acad. Sci. U.S.A. 69:1408).

The purified *Gaussia* polyA-mRNA is then subjected to a cDNA synthesis reaction to generate a cDNA library from total polyA-mRNA. Briefly, reverse transcriptase is used to extend an annealed polydT primer to generate an RNA/DNA duplex. The RNA strand is then digested using an RNase, e.g.,
20 RNase H, and following second-strand synthesis, the cDNA molecules are blunted-ended with S1 nuclease or other appropriate nuclease. The resulting double-stranded cDNA fragments can be ligated directly into a suitable expression vector or, alternatively, oligonucleotide linkers encoding restriction endonuclease sites can be ligated to the 5'-ends of the cDNA molecules to
25 facilitate cloning of the cDNA fragments.

b. Construction of cDNA expression libraries

The best characterized vectors for the construction of cDNA expression libraries are lambda vectors. Lambda-based vectors tolerate cDNA inserts of about 12 kb and provide greater ease in library screening, amplification and
30 storage compared to standard plasmid vectors. Presently preferred vectors for the preparation of *Gaussia* cDNA (and the other libraries herein) expression libraries are the Lambda, Uni-Zap, Lambda-Zap II or Lambda-ZAP

-83-

Express/EcoRI/XhoI vectors, which are known to those of skill in the art (e.g., see U.S. Pat. No. 5,128,256), and are also commercially available (Stratagene, La Jolla, CA).

Generally, the Lambda-Zap vectors combine the high efficiency of a
5 bacteriophage lambda vector systems with the versatility of a plasmid system. Fragments cloned into these vectors can be automatically excised using a helper phage and recircularized to generate subclones in the pBK-derived phagemid. The pBK phagemid carries the ampicillin -resistance gene (AMP^R) for selection in bacteria and G418 selection in eukaryotic cells or may contain the β -lactamase
10 resistance gene. Expression of the recombinant polypeptide is under the control of the *lacZ* promoter in bacteria and the CMV promoter in eukaryotes.

More specifically, these lambda-based vectors are composed of an initiator-terminator cassette containing the plasmid system, e.g., a the well known pBK Bluescript derivative (available from Stratagene), bracketed by the
15 right and left arm of the bacteriophage lambda. The lambda arms allow for efficient packaging of replicated DNA whereas the excisable initiator-terminator cassette allows for easy cloning of the cDNA fragments and the generation of a plasmid library without the need for additional subcloning.

When used herein, cDNA fragments are inserted into the multiple cloning
20 site contained within the initiator-terminator cassette of the Lambda-Zap vector to create a set of cDNA expression vectors. The set of cDNA expression vectors is allowed to infect suitable *E. coli* cells, followed by co-infection with a filamentous helper phage. Within the cell, trans-acting proteins encoded by the helper phage, e.g., the gene II protein of M13, recognize two separate domains
25 positioned within the lambda arms of the vector and introduce single-stranded nicks flanking the initiator-terminator cassette. Upon a subsequent round of DNA synthesis, a new DNA strand is synthesized that displaces the existing nick strand liberating the initiator-terminator cassette. The displaced strand is then circularized, packaged as filamentous phage by the helper proteins and
30 excreted from the cell. The BK plasmid containing the cDNA is recovered by infecting an F' strain of *E. coli* and plating the infected cells on solid medium supplemented with ampicillin for the selection of pBK-containing cells.

-84-

The *Gaussia* cDNA expression library can be screened using a variety of methods known to those of skill in the art. For example, identification of *Gaussia* luciferase may be achieved using a functional screening method by observing colonies visually for emission of blue light or by observing light emission using one or more bandpass filter.

3. Isolation and identification of DNA encoding *Gaussia* luciferase

DNA encoding a *Gaussia* luciferase may be isolated using methods described herein, or by using other methods known to those of skill in the art. As described in detail below, a *Gaussia* λ Uni-Zap cDNA expression plasmid library was prepared, transformed into competent *E. coli* cells and plated onto modified L-broth plates containing carbon black to absorb background fluorescence (e.g., see EXAMPLES).

Transformants were sprayed with a solution containing IPTG (isopropyl β -D-thiogalactopyranoside; see, et al. Nakamura et al. (1979) Cell 18:1109-1117) to induce expression of the recombinant *Gaussia* luciferase from the heterologous DNA. Other induction systems may also be used. Preferred promoter regions are those that are inducible and functional in *E. coli* or early genes in vectors of viral origin. Examples of suitable inducible promoters and promoter regions include, but are not limited to: the *E. coli* lac operator responsive to isopropyl β -D-thiogalactopyranoside (IPTG; see, et al. Nakamura et al. (1979) Cell 18:1109-1117); the metallothionein promoter metal-regulatory-elements responsive to heavy-metal (e.g., zinc) induction (see, e.g., U.S. Patent No. 4,870,009 to Evans et al.); the phage T7lac promoter responsive to IPTG (see, e.g., U.S. Patent No. 4,952,496; and Studier et al. (1990) Meth. Enzymol. 185:60-89) and the TAC promoter. Other promoters include, but are not limited to, the T7 phage promoter and other T7-like phage promoters, such as the T3, T5 and SP6 promoters, the trp, lpp, and lac promoters, such as the lacUV5, from *E. coli*; the P10 or polyhedrin gene promoter of baculovirus/insect cell expression systems (see, e.g., U.S. Patent Nos. 5,243,041, 5,242,687, 5,266,317, 4,745,051, and 5,169,784) and inducible promoters from other eukaryotic expression systems.

-85-

Particularly preferred plasmids for transformation of E. coli cells include the pET expression vectors (see, U.S patent 4,952,496; available from NOVAGEN, Madison, WI; see, also literature published by Novagen describing the system). Such plasmids include, pET 34 (see Fig. 1), pET 11a, which
5 contains the T7lac promoter, T7 terminator, the inducible E. coli lac operator, and the lac repressor gene; pET 12a-c, which contains the T7 promoter, T7 terminator, and the E. coli ompT secretion signal; and pET 15b (NOVAGEN, Madison, WI), which contains a His-TagTM leader sequence) for use in
10 purification with a His column and a thrombin cleavage site that permits cleavage following purification over the column; the T7-lac promoter region and the T7 terminator. Plasmid pET 34 further includes the CBD to aid in purification.

Particularly preferred plasmids for transformation of E. coli cells include the pET expression vectors (see, U.S patent 4,952,496; available from
15 NOVAGEN, Madison, WI). For example, the plasmid pET34-LIC is a prokaryotic expression vector that contains a multiple cloning site for inserting heterologous DNA templates downstream from a bacteriophage T7 promoter. Transformation into a bacterial host that expresses T7 RNA polymerase, e.g., E. coli strain BL21(DE3), results in high level, recombinant expression of the heterologous
20 protein. DNA encoding the *Gaussia* luciferase has been inserted into the pET34 vector as a fusion with the cellulose binding domain (CBD; see, SEQ ID Nos. 21 and 22), and expressed in E. coli host cells.

Over 120 different CBD sequences have been identified and grouped into at least 10 families on the basis of sequence similarities (Tomme et al. (1995)
25 in Enzymatic Degradation of Insoluble Polysaccharides; Saddler, J. M., and Penner, M., Eds.; American Chemical Society, Washington, D.C; pp 142-161). The CBD_{clos}-Tag sequence is derived from the Cellulose-Binding Protein A (CbpA) of
Clostridium cellulovorans (Goldstein et al. (1993) J. Bacteriol. 175:5762-5768)
30 and has a high affinity for crystalline cellulose.

To identify luciferase-expressing clones, transformants, grown on black agar, were sprayed with coelentraine.

-86-

Expression was apparent because of the resulting colonies that emit an intense blue-green light. Glowing colonies were selected. The nucleotide sequence of the cDNA insert of a blue light-emitting transformant was determined (e.g., see SEQ ID No. 19). As described in herein the 765 DNA
5 insert encodes a 185 amino acid polypeptide.

ISOLATION AND IDENTIFICATION OF NUCLEIC ACID ENCODING *Renilla* PROTEINS

1. Isolation of specimens of the genus *Renilla*

Specimens of *Renilla* are readily available from the oceans of the world,
10 including the Gulf of Mexico, Pacific Ocean and Atlantic Ocean. *Renilla* typically live on the ocean bottom at about 30 to 100 feet deep and can be easily collected by dragging. For example, specimens of *R. kollikeri* can be obtained off the coast of California or Baja, Mexico. Alternatively, live specimens of *Renilla* may be purchased from a commercial supplier (e.g., Gulf
15 Marine Incorporated, Panacea, Fla). Upon capture or receipt, the specimens are washed thoroughly and may also be dissected to enrich for light-emitting tissues. The whole organisms or dissected tissues are then snap frozen and stored in liquid nitrogen.

As described in detail in the examples below, the frozen tissues were
20 used as a source to isolate nucleic acids encoding *Renilla mulleri* GFP and luciferase (e.g., see SEQ ID No. 15 and SEQ ID No. 17, respectively).

2. Preparation of *Renilla* cDNA expression libraries

Renilla cDNA expression libraries may be prepared from intact RNA following the methods described herein or by other methods known to those of
25 skill the art (e.g., see Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.; U.S. Patent No. 5,292,658).

Typically, the preparation of cDNA libraries includes the isolation of polyadenylated RNA from the selected organism followed by single-strand DNA
30 synthesis using reverse transcriptase, digestion of the RNA strand of the DNA/RNA hybrid and subsequent conversion of the single-stranded DNA to double stranded cDNA.

-87-

a. RNA isolation and cDNA synthesis

Whole *Renilla* or dissected *Renilla* tissues can be used a source of total cytoplasmic RNA for the preparation of *Renilla* cDNA. Total intact RNA can be isolated from crushed *Renilla* tissue, for example, by using a modification of methods generally known in the art (e.g., see Chirgwin *et al.* (1970) Biochemistry 18:5294-5299). After isolating total cellular RNA, polyadenylated RNA species are then easily separated from the nonpolyadenylated species using affinity chromatography on oligodeoxythymidylate cellulose columns, (e.g., as described by Aviv *et al.*, (1972) Proc. Natl. Acad. Sci. U.S.A. 69:1408).

The purified *Renilla* polyA-mRNA is then subjected to a cDNA synthesis reaction to generate a cDNA library from total polyA-mRNA. Briefly, reverse transcriptase is used to extend an annealed polydT primer to generate an RNA/DNA duplex. The RNA strand is then digested using an RNase, e.g., RNase H, and following second-strand synthesis, the cDNA molecules are blunted-ended with S1 nuclease or other appropriate nuclease. The resulting double-stranded cDNA fragments can be ligated directly into a suitable expression vector or, alternatively, oligonucleotide linkers encoding restriction endonuclease sites can be ligated to the 5'-ends of the cDNA molecules to facilitate cloning of the cDNA fragments.

b. Construction of cDNA expression libraries

The best characterized vectors for the construction of cDNA expression libraries are lambda vectors. Lambda-based vectors tolerate cDNA inserts of about 12 kb and provide greater ease in library screening, amplification and storage compared to standard plasmid vectors. Presently preferred vectors for the preparation of *Renilla* cDNA expression libraries are the Lambda, Uni-Zap, Lambda-Zap II or Lambda-ZAP Express/EcoRI/XhoI vectors, which are known to those of skill in the art (e.g., see U.S. Pat. No. 5,128,256), and are also commercially available (Stratagene, La Jolla, CA).

Generally, the Lambda-Zap vectors combine the high efficiency of a bacteriophage lambda vector systems with the versatility of a plasmid system. Fragments cloned into these vectors can be automatically excised using a helper

-88-

phage and recircularized to generate subclones in the pBK-derived phagemid. The pBK phagemid carries the neomycin-resistance gene for selection in bacteria and G418 selection in eukaryotic cells or may contain the β -lactamase resistance gene. Expression of the recombinant polypeptide is under the control of the *lacZ* promoter in bacteria and the CMV promoter in eukaryotes.

More specifically, these lambda-based vectors are composed of an initiator-terminator cassette containing the plasmid system, e.g., a pBK Bluescript derivative (Stratagene, San Diego), bracketed by the right and left arm of the bacteriophage lambda. The lambda arms allow for efficient packaging of replicated DNA whereas the excisable initiator-terminator cassette allows for easy cloning of the cDNA fragments and the generation of a plasmid library without the need for additional subcloning.

When used herein, cDNA fragments are inserted into the multiple cloning site contained within the initiator-terminator cassette of the Lambda-Zap vector to create a set of cDNA expression vectors. The set of cDNA expression vectors is allowed to infect suitable *E. coli* cells, followed by co-infection with a filamentous helper phage. Within the cell, trans-acting proteins encoded by the helper phage, e.g., the gene II protein of M13, recognize two separate domains positioned within the lambda arms of the vector and introduce single-stranded nicks flanking the initiator-terminator cassette. Upon a subsequent round of DNA synthesis, a new DNA strand is synthesized that displaces the existing nick strand liberating the initiator-terminator cassette. The displaced strand is then circularized, packaged as filamentous phage by the helper proteins and excreted from the cell. The BK plasmid containing the cDNA is recovered by infecting an F' strain of *E. coli* and plating the infected cells on solid medium supplemented with kanamycin for the selection of pBK-containing cells.

The *Renilla* cDNA expression library can be screened using a variety of methods known to those of skill in the art. For example, identification of *Renilla* GFP may be achieved using a functional screening method employing blue light and observing colonies visually for emission of green fluorescence or by observing light emission using one or more bandpass filter.

3. Isolation and identification of DNA encoding *Renilla* GFP

-89-

DNA encoding a *Renilla* GFP may be isolated using methods described herein, or by using other methods known to those of skill in the art. As described in detail below, a *R. mulleri* λ Uni-Zap cDNA expression plasmid library was prepared, transformed into competent *E. coli* cells and plated onto
5 modified L-broth plates containing carbon black to absorb background fluorescence (e.g., see EXAMPLE 4). Transformants were sprayed with a solution containing IPTG to induce expression of the recombinant *Renilla* GFP from the heterologous cDNA. To identify GFP expressing clones, transformants were placed in blue light, preferably 470 to 490 nm light, and colonies that
10 emitted green fluorescence were isolated and grown in pure culture.

The nucleotide sequence of the cDNA insert of a green fluorescent transformant was determined (e.g., see SEQ ID No. 15). As described in EXAMPLE 4, the 1,079 cDNA insert encodes a 238 amino acid polypeptide that is only 23.5 % identical to *A. victoria* GFP, the only other GFP that has been
15 characterized at the molecular level. The recombinant protein exhibits excitation and emission spectra similar to those reported for live *Renilla* species.

4. Isolation and identification of DNA encoding *Renilla* luciferase

The above-described *R. mulleri* cDNA expression library was also used to
20 clone DNA encoding a *R. mulleri* luciferase (e.g., see EXAMPLE 5). Single colony transformants were grown on modified L-broth plates containing carbon black and expression from the heterologous DNA was induced with IPTG, essentially as described above. After allowing time for expression, the transformants were sprayed with coelenterazine and screened for those colonies
25 that emit blue light. Light-emitting colonies were isolated and grown in pure culture.

The nucleotide sequence of the cDNA insert contained in the light-emitting transformant was determined. As described in EXAMPLE 5, the 1,217 cDNA insert encodes a 311 amino acid polypeptide. The recombinant protein
30 exhibits excitation and emission spectra similar to those reported for live *Renilla* species.

-90-

D. NUCLEIC ACID PROBES AND METHODS FOR ISOLATING AND CLONING OF LUCIFERASE- and GFP-ENCODING NUCLEIC ACIDS FROM OTHER SPECIES

Gaussia

5 The nucleic acid exemplified herein that encodes the *Gaussia* luciferase may be used as a source of probes for isolating luciferases from other *Gaussia* species. Any suitable probe based upon the exemplified sequence of nucleotides may be used in any method. Such probe should hybridize under conditions of at least low stringency, more preferably moderate stringency and
10 most preferably high stringency to related nucleic acids in a suitable *Gaussia* library.

Also provided herein are specific nucleic acid probes for isolating and cloning luciferase-encoding nucleic acid from other species of *Gaussia*. Typically the nucleic acid probes are degenerate probes, which are then used
15 as hybridization probes to screen cDNA libraries prepared from the selected *Gaussia* species to obtain a DNA clone encoding a full-length *Gaussia* luciferase.

Preferred nucleic acid probes are designed to be degenerate probes of at least 14 nucleotides, preferably 16 to 30 nucleotides, that are based on these conserved amino acid positions. For example, particularly preferred regions for
20 designing probes are based on amino acids 1 to 185, set forth in SEQ ID No. 20. In other preferred embodiments, the nucleic acid probes encoding the above-described preferred amino acid regions are selected among the sequence of nucleotides encoding these regions set forth in SEQ ID NO. 19.

Alternatively, peptides corresponding to these amino acid positions can
25 be prepared and used as immunogens to immunize animals to produce *Gaussia* luciferase-specific polyclonal or monoclonal antibodies using methods well known to those of skill in the art (e.g., see Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). The antibodies can be used to screen cDNA expression libraries,
30 such as those prepared following the methods described herein, to identify clones expressing a partial or full-length clones.

-91-

**NUCLEIC ACID PROBES AND METHODS FOR ISOLATING AND
CLONING OF GFP-ENCODING NUCLEIC ACIDS FROM OTHER
SPECIES OF *Renilla***

The nucleic acid exemplified herein that encodes the *Renilla mulleri* GFP
5 may be used as a source of probes for isolating GFPs from other *Renilla* species.
Any suitable probe based upon the exemplified sequence of nucleotides may be
used in any method. Such probe should hybridize under conditions of low
stringency to related nucleic acid in a suitable *Renilla* library.

Also provided herein are specific nucleic acid probes for isolating and
10 cloning GFP-encoding nucleic acid from other species of *Renilla*. These probes
are based on regions of the *Renilla* GFP protein that are shared amongst
members of the *Renilla* genus (see Figure 1). Typically the nucleic acid probes
are degenerate probes, which are then used as hybridization probes to screen
cDNA libraries prepared from the selected *Renilla* species to obtain a DNA clone
15 encoding a full-length *Renilla* GFP.

To elucidate regions of the GFP that are shared amongst of *Renilla*
species, purified *Renilla reniformis* GFP was subjected to specific chemical and
proteolytic degradation, e.g., trypsin and Proteinase Q, to produce a variety of
short peptides for analysis and the amino acid sequence of the *Renilla reniformis*
20 peptides was determined.

Figure 1 displays an alignment of the deduced amino acid sequence of
Renilla mulleri green fluorescent protein and the amino acid sequence
determined for the isolated *Renilla reniformis* GFP peptides. Although the two
species are closely related, the amino acid sequences of the *Renilla* GFPs are
25 different. This difference, however, can be exploited to construct specific
probes because there are highly conserved regions. The *R. mulleri* and *R.*
reniformis sequences are identical at 103 of 187 residues present in peptides of
sufficient length to yield satisfactory alignments.

Certain regions of the two amino acid sequences exhibit a high degree of
30 conservation. For instance, 18 of 19 amino acids corresponding to positions 51
to 69 of the *Renilla mulleri* sequence are identical between the two *Renilla*
GFPs, including a contiguous stretch of 16 identical amino acid residues which

-92-

correspond to amino acid positions 51 to 65. Also, as shown in Figure 1, *Renilla reniformis* GFP (e.g., see SEQ ID No. 20) shares a fairly high degree of sequence similarity with the amino acid residues corresponding to amino acids 81 to 106 of the *R. mulleri* sequence (60.9 %; 18 of 26 identical amino acids).

5 Therefore, these regions provide the sequence for construction of probes.

Preferred nucleic acid probes are designed to be degenerate probes of at least 14 nucleotides, preferably 16 to 30 nucleotides, that are based on these conserved amino acid positions. For example, particularly preferred regions for designing probes are based on amino acids 51 to 68, 82 to 98 and 198 to 208
10 set forth in SEQ ID No. 16, amino acid sequence set forth in SEQ ID No. 20, amino acids 9-20 set forth in SEQ ID No. 21 and amino acids 39-53 set forth in SEQ ID No. 23. In other embodiments, the nucleic acid probes encoding the above-described preferred amino acid regions are selected among the sequence of nucleotides encoding these regions set forth in SEQ ID NO. 15. These
15 degenerate nucleic acid probes can be used as hybridization probes for the isolation and cloning of GFP-encoding DNA in *Renilla reniformis* and other species. Alternatively or in addition, these probes may be used as primers in nucleic acid amplification reactions.

Alternatively, peptides corresponding to these amino acid positions can
20 be prepared and used as immunogens to immunize animals to produce *Renilla* GFP-specific polyclonal or monoclonal antibodies using methods well known to those of skill in the art (e.g., see Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). The antibodies can be used to screen cDNA expression libraries, such as
25 those prepared following the methods described herein, to identify clones expressing a partial or full-length clone encompassing all or a portion of amino acid residues 51 to 69 of the *Renilla mulleri* GFP (e.g., see Figure 1; SEQ ID Nos. 15 and 16).

-93-

Other species

Similar methods may be used with the nucleic acids provided herein that encode the *Ptilosarcus* and *Pleuromamma* proteins.

E. RECOMBINANT EXPRESSION OF PROTEINS**5 *Gaussia*****1. DNA encoding *Gaussia* proteins**

As described above, DNA encoding a *Gaussia* luciferase can be isolated from natural sources, synthesized based on *Gaussia* nucleic acid sequences — provided herein or prepared using a number of recombinant DNA cloning and
10 amplification techniques, e.g., polymerase chain reaction (PCR).

In preferred embodiments, the DNA fragment encoding a *Gaussia* luciferase has the sequence of amino acids set forth in SEQ ID No. 20. In more preferred embodiments, the DNA fragment encodes the sequence of amino acids encoded by nucleotides 37-591 of the sequence of nucleotides set forth in
15 SEQ ID No. 19.

2. DNA constructs for recombinant production of *Gaussia* proteins

DNA is introduced into a plasmid for expression in a desired host. In preferred embodiments, the host is a bacterial host. The sequences of
20 nucleotides in the plasmids that are regulatory regions, such as promoters and operators, are operationally associated with one another for transcription of the sequence of nucleotides that encode a *Gaussia* luciferase. The sequence of nucleotides encoding the *Gaussia* luciferase may also include DNA encoding a secretion signal, whereby the resulting peptide is a precursor of the *Gaussia*
25 luciferase

In preferred embodiments the DNA plasmids also include a transcription terminator sequence. The promoter regions and transcription terminators are each independently selected from the same or different genes.

A wide variety of multipurpose vectors suitable for the expression of
30 heterologous proteins are known to those of skill in the art and are commercially available. Expression vectors containing inducible promoters or constitutive promoters that are linked to regulatory regions are preferred. Such promoters

-94-

include, but are not limited to, the T7 phage promoter and other T7-like phage promoters, such as the T3, T5 and SP6 promoters, the trp, lpp, tet and lac promoters, such as the lacUV5, from E. coli; the SV40 promoter; the P10 or polyhedron gene promoter of baculovirus/insect cell expression systems, 5 retroviral long-terminal repeats and inducible promoters from other eukaryotic expression systems.

3. Host organisms for recombinant production of *Gaussia* proteins

Host organisms include those organisms in which recombinant 10 production of heterologous proteins have been carried out, such as, but not limited to, bacteria (for example, E. coli), yeast (for example, Saccharomyces cerevisiae and Pichia pastoris), fungi, baculovirus/insect systems, amphibian cells, mammalian cells, plant cells and insect cells. Presently preferred host organisms are strains of bacteria or yeast. Most preferred host organisms are 15 strains of E. coli or Saccharomyces cerevisiae.

4. Methods for recombinant production of *Gaussia* proteins

The DNA encoding a *Gaussia* luciferase is introduced into a plasmid in operative linkage to an appropriate promoter for expression of polypeptides in a selected host organism. The DNA fragment encoding the *Gaussia* luciferase 20 may also include a protein secretion signal that functions in the selected host to direct the mature polypeptide into the periplasm or culture medium. The resulting luciferase can be purified by methods routinely used in the art, including methods described hereinafter in the Examples.

Methods of transforming suitable host cells, preferably bacterial cells, 25 and more preferably E. coli cells, as well as methods applicable for culturing said cells containing a gene encoding a heterologous protein, are generally known in the art. See, for example, Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

30 Once the *Gaussia*-encoding nucleic acid molecule has been introduced into the host cell, the desired protein is produced by subjecting the host cell to conditions under which the promoter is induced, whereby the operatively linked

-95-

DNA is transcribed. The cellular extracts of lysed cells containing the protein may be prepared and the resulting "clarified lysate" employed as a source of the luciferase. Alternatively, the lysate may be subjected to additional purification steps (e.g., ion exchange chromatography or immunoaffinity chromatography) to further enrich the lysate or provide a homogeneous source of the purified enzyme (see e.g., U.S. Patent Nos. 5,292,658 and 5,418,155).

Renilla

1. DNA encoding *Renilla* proteins —

As described above, DNA encoding a *Renilla* GFP or *Renilla* luciferase can be isolated from natural sources, synthesized based on *Renilla* sequences provided herein or prepared using a number of recombinant DNA cloning and amplification techniques, e.g., polymerase chain reaction (PCR).

In preferred embodiments, the DNA fragment encoding a *Renilla* GFP has the sequence of amino acids set forth in SEQ ID No. 16. In more preferred embodiments, the DNA fragment encodes the sequence of amino acids encoded by nucleotides 259-972 of the sequence of nucleotides set forth in SEQ ID No. 15.

In preferred embodiments, the DNA fragment encoding a *Renilla* luciferase has the sequence of amino acids set forth in SEQ ID No. 18. In more preferred embodiments, the DNA fragment encodes the sequence of amino acids encoded by nucleotides 31-963 of the sequence of nucleotides set forth in SEQ ID No. 17.

2. DNA constructs for recombinant production of *Renilla* proteins

DNA is introduced into a plasmid for expression in a desired host. In preferred embodiments, the host is a bacterial host. The sequences of nucleotides in the plasmids that are regulatory regions, such as promoters and operators, are operationally associated with one another for transcription of the sequence of nucleotides that encode a *Renilla* GFP or luciferase. The sequence of nucleotides encoding the FGF mutein may also include DNA encoding a secretion signal, whereby the resulting peptide is a precursor of the *Renilla* GFP.

-96-

In preferred embodiments the DNA plasmids also include a transcription terminator sequence. The promoter regions and transcription terminators are each independently selected from the same or different genes.

A wide variety of multipurpose vectors suitable for the expression of heterologous proteins are known to those of skill in the art and are commercially available. Expression vectors containing inducible promoters or constitutive promoters that are linked to regulatory regions are preferred. Such promoters include, but are not limited to, the T7 phage promoter and other T7-like phage promoters, such as the T3, T5 and SP6 promoters, the trp, lpp, tet and lac promoters, such as the lacUV5, from E. coli; the SV40 promoter; the P10 or polyhedron gene promoter of baculovirus/insect cell expression systems, retroviral long-terminal repeats and inducible promoters from other eukaryotic expression systems.

Particularly preferred vectors for recombinant expression of *Renilla mulleri* in prokaryotic organisms are lac- and T7 promoter-based vectors, such as the well known Bluescript vectors, which are commercially available (Stratagene, La Jolla, CA).

3. Host organisms for recombinant production of *Renilla* proteins

Host organisms include those organisms in which recombinant production of heterologous proteins have been carried out, such as, but not limited to, bacteria (for example, E. coli), yeast (for example, Saccharomyces cerevisiae and Pichia pastoris), fungi, baculovirus/insect systems, amphibian cells, mammalian cells, plant cells and insect cells. Presently preferred host organisms are strains of bacteria or yeast. Most preferred host organisms are strains of E. coli or Saccharomyces cerevisiae.

4. Methods for recombinant production of *Renilla* proteins

The DNA encoding a *Renilla* GFP or *Renilla mulleri* luciferase is introduced into a plasmid in operative linkage to an appropriate promoter for expression of polypeptides in a selected host organism. The DNA fragment encoding the *Renilla* GFP or luciferase may also include a protein secretion signal that functions in the selected host to direct the mature polypeptide into the

-97-

periplasm or culture medium. The resulting *Renilla* GFP or luciferase can be purified by methods routinely used in the art, including methods described hereinafter in the Examples.

5 Methods of transforming suitable host cells, preferably bacterial cells, and more preferably *E. coli* cells, as well as methods applicable for culturing said cells containing a gene encoding a heterologous protein, are generally known in the art. See, for example, Sambrook *et al.* (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

10 Once the *Renilla*-encoding DNA fragment has been introduced into the host cell, the desired *Renilla* GFP is produced by subjecting the host cell to conditions under which the promoter is induced, whereby the operatively linked DNA is transcribed. The cellular extracts of lysed cells containing the protein may be prepared and the resulting "clarified lysate" was employed as a source
15 of recombinant *Renilla* GFP or *Renilla mulleri* luciferase. Alternatively, the lysate may be subjected to additional purification steps (e.g., ion exchange chromatography or immunoaffinity chromatography) to further enrich the lysate or provide a homogeneous source of the purified enzyme (see *e.g.*, U.S. Patent Nos. 5,292,658 and 5,418,155).

20 **F. RECOMBINANT CELLS EXPRESSING HETEROLOGOUS NUCLEIC ACID ENCODING A LUCIFERASES AND GFPs**

These cells, vectors and methods are exemplified with respect to *Renilla* and *Gaussia*. The same cells, vectors and methods may be used for expressing the *Pleuromamma* and *Ptilosarcus* proteins.

25 ***Gaussia***

Recombinant cells containing heterologous nucleic acid encoding a *Gaussia* luciferase are provided. In preferred embodiments, the recombinant cells express the encoded *Gaussia* luciferase which is functional and non-toxic to the cell. In more preferred embodiments, the *Gaussia* luciferase contains the
30 amino acid sequence set forth in SEQ ID No. 20.

In certain embodiments, the recombinant cells may also include heterologous nucleic acid encoding another component(s) of a bioluminescence-

-98-

generating system, preferably a fluorescent protein. In preferred embodiments, the nucleic acid encoding the bioluminescence-generating system component is isolated from the species *Aequorea*, *Vargula* or *Renilla*. In more preferred embodiments, the additional bioluminescence-generating system component is a

5 *Renilla mulleri* or *reniformis* GFP.

The *Renilla* GFP and GFP peptides can be isolated from natural sources or isolated from a prokaryotic or eukaryotic cell transfected with nucleic acid that encodes the *Renilla* GFP and/or GFP peptides, such as those described above.

10 Exemplary cells include bacteria (e.g., *E. coli*), plant cells, cells of mammalian origin (e.g., COS cells, mouse L cells, Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK) cells, African green monkey cells and other such cells known to those of skill in the art), amphibian cells (e.g., *Xenopus laevis* oocytes), yeast cells (e.g., *Saccharomyces cerevisiae*, *Pichia*

15 *pastoris*), and the like. Exemplary cells for expressing injected RNA transcripts include *Xenopus laevis* oocytes. Eukaryotic cells that are preferred for transfection of DNA are known to those of skill in the art or may be empirically identified, and include HEK293 (which are available from ATCC under accession #CRL 1573); Ltk⁻ cells (which are available from ATCC under accession

20 #CCL1.3); COS-7 cells (which are available from ATCC under accession #CRL 1651); and DG44 cells (dhfr⁻ CHO cells; see, e.g., Urlaub et al. (1986) Cell. Molec. Genet. 12: 555). Presently preferred cells include strains of bacteria and yeast.

The recombinant cells that contain the heterologous DNA encoding the

25 *Gaussia* luciferase are produced by transfection with DNA encoding a *Gaussia* luciferase or by introduction of RNA transcripts of DNA encoding *Gaussia* proteins using methods well known to those of skill in the art. The DNA may be introduced as a linear DNA fragment or may be included in an expression vector for stable or transient expression of the encoding DNA.

30 Heterologous DNA may be maintained in the cell as an episomal element or may be integrated into chromosomal DNA of the cell. The resulting recombinant cells may then be cultured or subcultured (or passaged, in the case

-99-

of mammalian cells) from such a culture or a subculture thereof. Also, DNA may be stably incorporated into cells or may be transiently expressed using methods known in the art.

The recombinant cells can be used in a wide variety of cell-based assay methods, such as those methods described for cells expressing wild type or modified *A. victoria* GFPs or GFP fusion proteins (e.g., see U.S. Patent No. 5,625,048; International patent application Publication Nos. WO 95/21191; WO 96/23810; WO 96/27675; WO 97/26333; WO 97/28261; WO 97/41228; and WO 98/02571).

10 **RECOMBINANT CELLS EXPRESSING HETEROLOGOUS NUCLEIC ACID
ENCODING A *Renilla* GREEN FLUORESCENT PROTEIN AND/OR
LUCIFERASE**

Recombinant cells containing heterologous nucleic acid encoding a *Renilla* GFP are provided. In preferred embodiments, the recombinant cells
15 express the encoded *Renilla* GFP which is functional and non-toxic to the cell.

In certain embodiments, the recombinant cells may also include heterologous nucleic acid encoding a component of a bioluminescence-generating system, preferably a photoprotein or luciferase. In preferred
20 embodiments, the nucleic acid encoding the bioluminescence-generating system component is isolated from the species *Aequorea*, *Vargula* or *Renilla*. In more preferred embodiments, the bioluminescence-generating system component is a *Renilla mulleri* luciferase having the amino acid sequence set forth in SEQ ID No. 18.

Recombinant host cells containing heterologous nucleic acid encoding a
25 *Renilla mulleri* luciferase are also provided. In preferred embodiments, the heterologous nucleic acid encodes the sequence of amino acids as set forth in SEQ ID No. 18. In more preferred embodiments, the heterologous nucleic acid encodes the sequence of nucleotides set forth in SEQ ID No. 17.

Exemplary cells include bacteria (e.g., *E. coli*), plant cells, cells of
30 mammalian origin (e.g., COS cells, mouse L cells, Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK) cells, African green monkey cells and other such cells known to those of skill in the art), amphibian cells (e.g.,

-100-

Xenopus laevis oocytes), yeast cells (e.g., *Saccharomyces cerevisiae*, *Pichia pastoris*), and the like. Exemplary cells for expressing injected RNA transcripts include *Xenopus laevis* oocytes. Eukaryotic cells that are preferred for transfection of DNA are known to those of skill in the art or may be empirically identified, and include HEK293 (which are available from ATCC under accession #CRL 1573); Ltk⁻ cells (which are available from ATCC under accession #CCL1.3); COS-7 cells (which are available from ATCC under accession #CRL 1651); and DG44 cells (dhfr⁻ CHO cells; see, e.g., Urlaub et al. (1986) Cell. Molec. Genet. 12: 555). Presently preferred cells include strains of bacteria and yeast.

The recombinant cells that contain the heterologous DNA encoding the *Renilla* GFP are produced by transfection with DNA encoding a *Renilla* GFP or luciferase or by introduction of RNA transcripts of DNA encoding a *Renilla* proteins using methods well known to those of skill in the art. The DNA may be introduced as a linear DNA fragment or may be included in an expression vector for stable or transient expression of the encoding DNA.

Heterologous DNA may be maintained in the cell as an episomal element or may be integrated into chromosomal DNA of the cell. The resulting recombinant cells may then be cultured or subcultured (or passaged, in the case of mammalian cells) from such a culture or a subculture thereof. Also, DNA may be stably incorporated into cells or may be transiently expressed using methods known in the art.

The recombinant cells can be used in a wide variety of cell-based assay methods, such as those methods described for cells expressing wild type or modified *A. victoria* GFPs or GFP fusion proteins (e.g., see U.S. Patent No. 5,625,048; International patent application Publication Nos. WO 95/21191; WO 96/23810; WO 96/27675; WO 97/26333; WO 97/28261; WO 97/41228; and WO 98/02571).

G. Luciferases

Purified *Gaussia* luciferase and *Gaussia* luciferase peptides as wells as *Pleuromamma* and *Renilla mulleri* luciferases are provided. The luciferase is

-101-

produced by expressing the protein in selected host cells and isolating the resulting luciferase.

Nucleic acid encoding a *Renilla mulleri* luciferase is also provided. The nucleic acid is used to produce the encoded luciferase. Presently preferred
5 *Renilla mulleri* luciferase for use in compositions, combinations and methods has the amino acid sequence set forth in SEQ ID No. 18. The luciferase can be formulated for compositions and combinations that have a wide variety of end-use applications, such as those described herein.

H. *Renilla* and *Ptilosarcus* GFPs

10 Purified *Renilla* GFPs, particularly *Renilla mulleri* GFP, and purified *Renilla reniformis* GFP peptides are provided. Presently preferred *Renilla* GFP for use in the compositions herein is *Renilla mulleri* GFP having the sequence of amino acids set forth in SEQ ID No. 16. Presently preferred *Renilla reniformis* GFP
15 peptides are those containing the GFP peptides selected from the amino acid sequences set forth in SEQ ID Nos 19-23.

The *Renilla* GFP and GFP peptides can be isolated from natural sources or isolated from a prokaryotic or eukaryotic cell transfected with nucleic acid that encodes the *Renilla* GFP and/or GFP peptides, such as those described in Section F above.

20 I. COMPOSITIONS

As above, compositions and conjugates and methods of use are described with reference to *Gaussia* and *Renilla* proteins and nucleic acids. The same compositions and methods for preparation and use thereof are intended for use with *Pleuromamma* and *Ptilosarcus* proteins and nucleic acids.

25 1. *Gaussia* luciferase compositions

Compositions containing a *Gaussia* luciferase are provided. The compositions may also contain a *Renilla* GFP or GFP peptide. The compositions can take any of a number of forms, depending on the intended method of use therefor. In certain embodiments, the compositions are prepared for use in
30 bioluminescent novelty items, immunoassays or FRET and FET assays. The compositions may also be used in conjunction with multi-well assay devices containing integrated photodetectors (see,, e.g., copending U.S. application

-102-

Serial No. 08/990,103), for detection of tumors (see, e.g., U.S. application Serial No. 08/908,909, or in bioluminescent novelty items (see, U.S. application Serial Nos. 08/597,274 and 08/757,046.

These compositions can be used in a variety of methods and systems,
5 such as included in conjunction with diagnostic systems for the *in vivo* detection of neoplastic tissues and other tissues, such as those methods described in detail below. These methods and products include any known to those of skill in the art in which luciferase is used, including, but not limited to U.S. application Serial Nos. 08/757,046, 08/597,274 and 08/990,103, U.S.
10 Patent No. 5,625,048; International patent application Publication Nos. WO 95/21191; WO 96/23810; WO 96/27675; WO 97/26333; WO 97/28261; WO 97/41228; and WO 98/02571).

2. *Renilla* luciferase compositions

The DNA encoding the *Renilla mulleri* luciferase is used to produce the
15 encoded luciferase, which has diagnostic applications as well as use as a component of the bioluminescence generating systems as described herein, such as in beverages, and methods of diagnosis of neoplasia and in the diagnostic chips described herein. These methods and products include any known to those of skill in the art in which luciferase is used, including, but not
20 limited to, U.S. application Serial No. 08/757,046, 08/597,274 and 08/990,103, U.S. Patent No. 5,625,048; International patent application Publication Nos. WO 95/21191; WO 96/23810; WO 96/27675; WO 97/26333; WO 97/28261; WO 97/41228; and WO 98/02571).

In other embodiments, the *Renilla mulleri* luciferase and the remaining
25 components may be packaged as separate compositions, that, upon mixing, glow. For example, a composition containing *Renilla mulleri* luciferase may be provided separately from, and for use with, an a separate composition containing a bioluminescence substrate and bioluminescence activator. In another instance, luciferase and luciferin compositions may be separately
30 provided and the bioluminescence activator may be added after, or simultaneously with, mixing of the other two compositions.

-103-

3. *Renilla* GFP compositions

Compositions containing a *Renilla* GFP or GFP peptide are provided. The compositions can take any of a number of forms, depending on the intended method of use therefor. In certain embodiments, for example, the compositions
5 contain a *Renilla* GFP or GFP peptide, preferably *Renilla mulleri* GFP or *Renilla reniformis* GFP peptide, formulated for use in luminescent novelty items, immunoassays, FRET and FET assays. The compositions may also be used in conjunction with multi-well assay devices containing integrated photodetectors, such as those described herein.

10 Compositions that contain a *Renilla mulleri* GFP or GFP peptide and at least one component of a bioluminescence-generating system, preferably a luciferase, luciferin or a luciferase and a luciferin, are provided. In preferred embodiments, the luciferase/luciferin bioluminescence- generating system is selected from those isolated from: an insect system, a coelenterate system, a
15 ctenophore system, a bacterial system, a mollusk system, a crustacea system, a fish system, an annelid system, and an earthworm system. Presently preferred bioluminescence- generating systems are those isolated from *Renilla*, *Aequorea*, and *Vargula*.

In more preferred embodiments, the bioluminescence-generating system
20 component is a *Renilla mulleri* luciferase having the amino acid sequence set forth in SEQ ID No. 18. These compositions can be used in a variety of methods and systems, such as included in conjunction with diagnostic systems for the *in vivo* detection of neoplastic tissues and other tissues, such as those methods described in detail below.

25 These methods and products include any known to those of skill in the art in which luciferase is used, including, but not limited to U.S. application Serial No. 08/757,046, 08/597,274 and 08/990,103, U.S. Patent No. 5,625,048; International patent application Publication Nos. WO 95/21191; WO 96/23810; WO 96/27675; WO 97/26333; WO 97/28261; WO 97/41228; and
30 WO 98/02571).

-104-

4. Conjugates

The conjugates that are provided herein contain a targeting agent, such as a tissue specific or tumor specific monoclonal antibody or fragment thereof linked either directly or via a linker to a targeted agent, a *Renilla* GFP, *Renilla* 5 *mulleri* or *Gaussia* luciferase and other luciferases (including photoproteins or luciferase enzymes) or a luciferin. The targeted agent may be coupled to a microcarrier. The linking is effected either chemically, by recombinant expression of a fusion protein in instances when the targeted agent is a protein, and by combinations of chemical and recombinant expression. The targeting 10 agent is one that will preferentially bind to a selected tissue or cell type, such as a tumor cell surface antigen or other tissue specific antigen.

Methods for preparing conjugates are known to those of skill in the art. For example, aequorin that is designed for conjugation and conjugates containing such aequorin have been produced [see, e.g., International PCT 15 application No. WO 94/18342; see, also Smith *et al.* (1995) in American Biotechnology Laboratory]. Aequorin has been conjugated to an antibody molecule by means of a sulfhydryl-reacting binding agent (Stultz *et al.* (1992) *Use of Recombinant Biotinylated Apoaequorin from *Escherichia coli**. *Biochemistry* 31, 1433-1442). Such methods may be adapted for use herein to 20 produce the luciferase coupled to protein or other such molecules, which are useful as targeting agents. *Vargula* luciferase has also been linked to other molecules [see, e.g., Japanese application No. JP 5064583, March 19, 1993]. Such methods may be adapted for use herein to produce luciferase coupled to molecules that are useful as targeting agents.

25 The conjugates can be employed to detect the presence of or quantitate a particular antigen in a biological sample by direct correlation to the light emitted from the bioluminescent reaction.

As an alternative, a component of the bioluminescence generating system may be modified for linkage, such as by addition of amino acid residues 30 that are particularly suitable for linkage to the selected substrate. This can be readily effected by modifying the DNA and expressing such modified DNA to produce luciferase with additional residues at the N- or C-terminus.

-105-

Methods for preparing conjugates are known to those of skill in the art. For example, aequorin that is designed for conjugation and conjugates containing such aequorin have been produced [see, e.g., International PCT application No. WO 94/18342; see, also Smith et al. (1995) in American
5 Biotechnology Laboratory]. Aequorin has been conjugated to an antibody molecule by means of a sulfhydryl-reacting binding agent (Stultz et al. (1992) Use of Recombinant Biotinylated Apoaequorin from *Escherichia coli*. Biochemistry 31, 1433-1442). Such methods may be adapted for use herein to produce aequorin coupled to protein or other such molecules, which are useful
10 as targeting agents. *Vargula* luciferase has also been linked to other molecules [see, e.g., Japanese application No. JP 5064583, March 19, 1993]. Such methods may be adapted for use herein to produce aequorin coupled to protein or other such molecules, which are useful as targeting agents.

Aequorin-antibody conjugates have been employed to detect the
15 presence of or quantitate a particular antigen in a biological sample by direct correlation to the light emitted from the bioluminescent reaction.

Selection of the system depends upon factors such as the desired color and duration of the bioluminescence desired as well as the particular item. Selection of the targeting agent primarily depends upon the type and
20 characteristics of neoplasia or tissue to be visualized and the setting in which visualization will be performed. For example, the luciferase isolated from *Aristostomias* emits red light, which is particularly beneficial for preoperative diagnosis because the red light is detectable through tissue using a photomultiplier.

25 a. Linkers

Any linker known to those of skill in the art may be used herein. Other linkers are suitable for incorporation into chemically produced conjugates. Linkers that are suitable for chemically linked conjugates include disulfide bonds, thioether bonds, hindered disulfide bonds, and covalent bonds between
30 free reactive groups, such as amine and thiol groups. These bonds are produced using heterobifunctional reagents to produce reactive thiol groups on one or both of the polypeptides and then reacting the thiol groups on one

-106-

polypeptide with reactive thiol groups or amine groups to which reactive maleimido groups or thiol groups can be attached on the other. Other linkers include, acid cleavable linkers, such as bismaleimideoxy propane, acid labile-transferrin conjugates and adipic acid dihydrazide, that would be cleaved in more acidic intracellular compartments; cross linkers that are cleaved upon exposure to UV or visible light and linkers, such as the various domains, such as C_H1, C_H2, and C_H3, from the constant region of human IgG₁ (see, Batra *et al.* (1993) Molecular Immunol. 30:379-386). In some embodiments, several linkers may be included in order to take advantage of desired properties of each linker.

10

Chemical linkers and peptide linkers may be inserted by covalently coupling the linker to the TA and the targeted agent. The heterobifunctional agents, described below, may be used to effect such covalent coupling. Peptide linkers may also be linked by expressing DNA encoding the linker and TA, linker and targeted agent, or linker, targeted agent and TA as a fusion protein.

Flexible linkers and linkers that increase solubility of the conjugates are contemplated for use, either alone or with other linkers are contemplated herein.

Numerous heterobifunctional cross-linking reagents that are used to form covalent bonds between amino groups and thiol groups and to introduce thiol groups into proteins, are known to those of skill in this art (see, *e.g.*, the PIERCE CATALOG, ImmunoTechnology Catalog & Handbook, 1992-1993, which describes the preparation of and use of such reagents and provides a commercial source for such reagents; see, also, *e.g.*, Cumber *et al.* (1992) Bioconjugate Chem. 3:397-401; Thorpe *et al.* (1987) Cancer Res. 47:5924-5931; Gordon *et al.* (1987) Proc. Natl. Acad. Sci. 84:308-312; Walden *et al.* (1986) J. Mol. Cell Immunol. 2:191-197; Carlsson *et al.* (1978) Biochem. J. 173: 723-737; Mahan *et al.* (1987) Anal. Biochem. 162:163-170; Wawryznaczak *et al.* (1992) Br. J. Cancer 66:361-366; Fattom *et al.* (1992) Infection & Immun. 60:584-589). These reagents may be used to form covalent bonds between the TA and targeted agent. These reagents include, but are not limited to: N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP;

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-107-

disulfide linker); sulfosuccinimidyl 6-[3-(2-pyridyldithio)propionamido]hexanoate (sulfo-LC-SPDP); succinimidylloxycarbonyl- α -methyl benzyl thiosulfate (SMBT, hindered disulfate linker); succinimidyl 6-[3-(2-pyridyldithio) propionamido]-hexanoate (LC-SPDP); sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC); succinimidyl 3-(2-pyridyldithio)butyrate (SPDB; hindered disulfide bond linker); sulfosuccinimidyl 2-(7-azido-4-methylcoumarin-3-acetamide) ethyl-1,3'-dithiopropionate (SAED); sulfo-succinimidyl 7-azido-4-methylcoumarin-3-acetate (SAMCA); sulfosuccinimidyl 6-[α -methyl- α -(2-pyridyldithio)toluamido]hexanoate (sulfo-LC-SMPT); 1,4-di-[3'-(2'-pyridyldithio)propionamido]butane (DPDPB); 4-succinimidylloxycarbonyl- α -methyl- α -(2-pyridylthio)toluene (SMPT, hindered disulfate linker); sulfosuccinimidyl 6-[α -methyl- α -(2-pyridyldithio)toluamido]hexanoate (sulfo-LC-SMPT); *m*-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS); *m*-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sulfo-MBS); N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB; thioether linker); sulfosuccinimidyl(4-iodoacetyl)amino benzoate (sulfo-SIAB); succinimidyl(4-(*p*-maleimidophenyl)butyrate (SMPB); sulfosuccinimidyl(4-(*p*-maleimidophenyl)butyrate (sulfo-SMPB); azidobenzoyl hydrazide (ABH).

Acid cleavable linkers, photocleavable and heat sensitive linkers may also be used, particularly where it may be necessary to cleave the targeted agent to permit it to be more readily accessible to reaction.

Acid cleavable linkers include, but are not limited to, bismaleimideoxy propane; and adipic acid dihydrazide linkers (see, e.g., Fattom et al. (1992) Infection & Immun. 60:584-589) and acid labile transferrin conjugates that contain a sufficient portion of transferrin to permit entry into the intracellular transferrin cycling pathway (see, e.g., Welhöner et al. (1991) J. Biol. Chem. 266:4309-4314).

Photocleavable linkers are linkers that are cleaved upon exposure to light (see, e.g., Goldmacher et al. (1992) Bioconj. Chem. 3:104-107, which linkers are herein incorporated by reference), thereby releasing the targeted agent upon exposure to light. Photocleavable linkers that are cleaved upon exposure to light are known (see, e.g., Hazum et al. (1981) in Pept., Proc. Eur.

-108-

Pept. Symp., 16th, Brunfeldt, K (Ed), pp. 105-110, which describes the use of a nitrobenzyl group as a photocleavable protective group for cysteine; Yen *et al.* (1989) *Makromol. Chem* 190:69-82, which describes water soluble photocleavable copolymers, including hydroxypropylmethacrylamide copolymer, glycine copolymer, fluorescein copolymer and methylrhodamine copolymer; Goldmacher *et al.* (1992) *Bioconj. Chem.* 3:104-107, which describes a cross-linker and reagent that undergoes photolytic degradation upon exposure to near UV light (350 nm); and Senter *et al.* (1985) *Photochem. Photobiol* 42:231-237, which describes nitrobenzyloxycarbonyl chloride cross linking reagents that produce photocleavable linkages), thereby releasing the targeted agent upon exposure to light. Such linkers would have particular use in treating dermatological or ophthalmic conditions that can be exposed to light using fiber optics. After administration of the conjugate, the eye or skin or other body part can be exposed to light, resulting in release of the targeted moiety from the conjugate. Such photocleavable linkers are useful in connection with diagnostic protocols in which it is desirable to remove the targeting agent to permit rapid clearance from the body of the animal.

b. Targeting Agents

Targeting agents include any agent that will interact with and localize the targeted agent cells in a tumor or specialized tissue [targeted tissue]. Such agents include any agent that specifically interacts with a cell surface protein or receptor that is present at sufficiently higher concentrations or amounts on the targeted tissue, whereby, when contacted with an appropriate bioluminescence generating reagent and activators produces light. These agents include, but are not limited to, growth factors, preferentially modified to not internalize, methotrexate, and antibodies, particularly, antibodies raised against tumor specific antigens. A plethora of tumor-specific antigens have been identified from a number of human neoplasms.

Anti-tumor Antigen Antibodies

Polyclonal and monoclonal antibodies may be produced against selected antigens. Alternatively, many such antibodies are presently available. An exemplary list of antibodies and the tumor antigen for which each has been

-109-

directed against is provided in U.S. application Serial No., which is incorporated by reference in its entirety. It is contemplated that any of the antibodies listed may be conjugated with a bioluminescence generating component following the methods provided herein.

- 5 Among the preferred antibodies for use in the methods herein are those of human origin or, more preferably, are humanized monoclonal antibodies. These are preferred for diagnosis of humans.

Preparation of the conjugates —

- Any method for linking proteins may be used. For example, methods for
10 linking a luciferase to an antibody is described in U.S. Patent No. 5,486,455. As noted above, the targeting agent and luciferin or luciferase may be linked directly, such as through covalent bonds, i.e., sulfhydryl bonds or other suitable bonds, or they may be linked through a linker. There may be more than one luciferase or luciferin per targeting agent, or more than one targeting agent per
15 luciferase or luciferin.

- Alternatively, an antibody, or F(Ab)₂ antigen-binding fragment thereof or other protein targeting agent may be fused (directly or via a linking peptide) to the luciferase using recombinant DNA technology. For example, the DNA encoding any of the anti-tumor antibodies of Table 3 may be ligated in the same
20 translational reading frame to DNA encoding any of the above-described luciferases, e.g., SEQ ID NOs. 1-14 and inserted into an expression vector. The DNA encoding the recombinant antibody-luciferase fusion may be introduced into an appropriate host, such as bacteria or yeast, for expression.

5. Formulation of the compositions for use in the diagnostic systems

- 25 In most embodiments, the *Renilla* GFPS and components of the diagnostic systems provided herein, such as *Renilla mulleri* luciferase, are formulated into two compositions: a first composition containing the conjugate; and a second composition containing the remaining components of the bioluminescence generating system. The compositions are formulated in any
30 manner suitable for administration to an animal, particularly a mammal, and more particularly a human. Such formulations include those suitable for topical,

-110-

local, enteric, parenteral, intracystal, intracutaneous, intravitreal, subcutaneous, intramuscular, or intravenous administration.

For example, the conjugates, which in preferred embodiments, are a
5 targeting agent linked to a luciferase (or photoprotein) are formulated for systemic or local administration. The remaining components are formulated in a separate second composition for topical or local application. The second composition will typically contain any other agents, such as spectral shifters that will be included in the reaction. It is preferred that the components of the
10 second composition are formulated in a time release manner or in some other manner that prevents degradation and/or interaction with blood components.

a. The first composition: formulation of the conjugates

As noted above, the conjugates either contain a luciferase or luciferin and a targeting agents. The preferred conjugates are formed between a
15 targeting agent and a luciferase, particularly the *Gaussia*, *Renilla mulleri* or *Pleuromamma* luciferase. The conjugates may be formulated into pharmaceutical compositions suitable for topical, local, intravenous and systemic application. Effective concentrations of one or more of the conjugates are mixed with a suitable pharmaceutical carrier or vehicle. The concentrations
20 or amounts of the conjugates that are effective requires delivery of an amount, upon administration, that results in a sufficient amount of targeted moiety linked to the targeted cells or tissue whereby the cells or tissue can be visualized during the surgical procedure. Typically, the compositions are formulated for single dosage administration. Effective concentrations and amounts may be
25 determined empirically by testing the conjugates in known in vitro and in vivo systems, such as those described here; dosages for humans or other animals may then be extrapolated therefrom.

Upon mixing or addition of the conjugate(s) with the vehicle, the resulting mixture may be a solution, suspension, emulsion or the like. The form
30 of the resulting mixture depends upon a number of factors, including the intended mode of administration and the solubility of the conjugate in the selected carrier or vehicle. The effective concentration is sufficient for targeting

-111-

a sufficient amount of targeted agent to the site of interest, whereby when combined with the remaining reagents during a surgical procedure the site will glow. Such concentration or amount may be determined based upon in vitro and/or in vivo data, such as the data from the mouse xenograft model for
5 tumors or rabbit ophthalmic model. If necessary, pharmaceutically acceptable salts or other derivatives of the conjugates may be prepared.

Pharmaceutical carriers or vehicles suitable for administration of the conjugates provided herein include any such carriers known to those skilled in the art to be suitable for the particular mode of administration.

10 In addition, the conjugates may be formulated as the sole pharmaceutically ingredient in the composition or may be combined with other active ingredients.

The conjugates can be administered by any appropriate route, for example, orally, parenterally, intravenously, intradermally, subcutaneously, or topically, in liquid, semi-liquid or solid form and are formulated in a manner
15 suitable for each route of administration. Intravenous or local administration is presently preferred. Tumors and vascular proliferative disorders, will typically be visualized by systemic, intradermal or intramuscular, modes of administration.

The conjugate is included in the pharmaceutically acceptable carrier in an
20 amount sufficient to produce detectable tissue and to not result in undesirable side effects on the patient or animal. It is understood that number and degree of side effects depends upon the condition for which the conjugates are administered. For example, certain toxic and undesirable side effects are tolerated when trying to diagnose life-threatening illnesses, such as tumors, that
25 would not be tolerated when diagnosing disorders of lesser consequence.

The concentration of conjugate in the composition will depend on absorption, inactivation and excretion rates thereof, the dosage schedule, and amount administered as well as other factors known to those of skill in the art. Typically an effective dosage should produce a serum concentration of active
30 ingredient of from about 0.1 ng/ml to about 50-1000 μ g/ml, preferably 50-100 μ g/ml. The pharmaceutical compositions typically should provide a dosage of from about 0.01 mg to about 100 - 2000 mg of conjugate, depending upon the

-112-

conjugate selected, per kilogram of body weight per day. Typically, for intravenous administration a dosage of about between 0.05 and 1 mg/kg should be sufficient. Local application for, such as visualization of ophthalmic tissues or local injection into joints, should provide about 1 ng up to 1000 μ g, preferably about 1 μ g to about 100 μ g, per single dosage administration. It is understood that the amount to administer will be a function of the conjugate selected, the indication, and possibly the side effects that will be tolerated. Dosages can be empirically determined using recognized models.

The active ingredient may be administered at once, or may be divided into a number of smaller doses to be administered at intervals of time. It is understood that the precise dosage and duration of administration is a function of the disease condition being diagnosed and may be determined empirically using known testing protocols or by extrapolation from in vivo or in vitro test data. It is to be noted that concentrations and dosage values may also vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed compositions.

Solutions or suspensions used for parenteral, intradermal, subcutaneous, or topical application can include any of the following components: a sterile diluent, such as water for injection, saline solution, fixed oil, polyethylene glycol, glycerine, propylene glycol or other synthetic solvent; antimicrobial agents, such as benzyl alcohol and methyl parabens; antioxidants, such as ascorbic acid and sodium bisulfite; chelating agents, such as ethylenediaminetetraacetic acid (EDTA); buffers, such as acetates, citrates and phosphates; and agents for the adjustment of tonicity such as sodium chloride or dextrose. Parental preparations can be enclosed in ampules, disposable syringes or multiple dose vials made of glass, plastic or other suitable material.

If administered intravenously, suitable carriers include physiological saline or phosphate buffered saline (PBS), and solutions containing thickening and

-113-

solubilizing agents, such as glucose, polyethylene glycol, and polypropylene glycol and mixtures thereof. Liposomal suspensions may also be suitable as pharmaceutically acceptable carriers. These may be prepared according to methods known to those skilled in the art.

- 5 The conjugates may be prepared with carriers that protect them against rapid elimination from the body, such as time release formulations or coatings. Such carriers include controlled release formulations, such as, but not limited to, implants and microencapsulated delivery systems, and biodegradable, biocompatible polymers, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, polyorthoesters, polylactic acid and others.

- 10 The conjugates may be formulated for local or topical application, such as for topical application to the skin and mucous membranes, such as in the eye, in the form of gels, creams, and lotions and for application to the eye or for intracisternal or intraspinal application. Such solutions, particularly those
15 intended for ophthalmic use, may be formulated as 0.01% -10% isotonic solutions, pH about 5-7, with appropriate salts. The ophthalmic compositions may also include additional components, such as hyaluronic acid. The conjugates may be formulated as aerosols for topical application (see, e.g., U.S. Patent Nos. 4,044,126, 4,414,209, and 4,364,923).

- 20 Also, the compositions for activation of the conjugate in vivo during surgical procedures may be formulated as an aerosol. These compositions contain the activators and also the remaining bioluminescence generating agent, such as luciferin, where the conjugate targets a luciferase, or a luciferase, where the conjugate targets a luciferin, such as coelenterazine.

- 25 If oral administration is desired, the conjugate should be provided in a composition that protects it from the acidic environment of the stomach. For example, the composition can be formulated in an enteric coating that maintains its integrity in the stomach and releases the active compound in the intestine. Oral compositions will generally include an inert diluent or an edible carrier and
30 may be compressed into tablets or enclosed in gelatin capsules. For the purpose of oral administration, the active compound or compounds can be incorporated with excipients and used in the form of tablets, capsules or

-114-

troches. Pharmaceutically compatible binding agents and adjuvant materials can be included as part of the composition.

Tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder, such as
5 microcrystalline cellulose, gum tragacanth and gelatin; an excipient such as starch and lactose, a disintegrating agent such as, but not limited to, alginic acid and corn starch; a lubricant such as, but not limited to, magnesium stearate; a glidant, such as, but not limited to, colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; and a flavoring agent such as
10 peppermint, methyl salicylate, and fruit flavoring.

When the dosage unit form is a capsule, it can contain, in addition to material of the above type, a liquid carrier such as a fatty oil. In addition, dosage unit forms can contain various other materials which modify the physical form of the dosage unit, for example, coatings of sugar and other enteric
15 agents. The conjugates can also be administered as a component of an elixir, suspension, syrup, wafer, chewing gum or the like. A syrup may contain, in addition to the active compounds, sucrose as a sweetening agent and certain preservatives, dyes and colorings and flavors.

The active materials can also be mixed with other active materials that
20 do not impair the desired action, or with materials that supplement the desired action, such as cis-platin for treatment of tumors.

Finally, the compounds may be packaged as articles of manufacture containing packaging material, one or more conjugates or compositions as provided herein within the packaging material, and a label that indicates the
25 indication for which the conjugate is provided.

b. The second composition

The second composition will include the remaining components of the bioluminescence generating reaction. In preferred embodiments in which these components are administered systemically, the remaining components include
30 the luciferin or substrate, and optionally additional agents, such as spectral shifters, particularly the GFPs provided herein. These components, such as the luciferin, can be formulated as described above for the conjugates. In some

-115-

embodiments, the luciferin or luciferase in this composition will be linked to a protein carrier or other carrier to prevent degradation or dissolution into blood cells or other cellular components.

For embodiments, in which the second composition is applied locally or topically, they can be formulated in a spray or aerosol or other suitable means for local or topical application.

In certain embodiments described herein, all components, except an activator are formulated together, such as by encapsulation in a time release formulation that is targeted to the tissue. Upon release the composition will have been localized to the desired site, and will begin to glow.

In practice, the two compositions can be administered simultaneously or sequentially. Typically, the first composition, which contains the conjugate is administered first, generally an hour or two before the surgery, and the second composition is then administered, either pre-operatively or during surgery.

The conjugates that are provided herein contain a targeting agent, such as a tissue specific or tumor specific monoclonal antibody or fragment thereof linked either directly or via a linker to a targeted agent, a luciferase (including photoproteins or luciferase enzymes) or a luciferin. The targeted agent may be coupled to a microcarrier. The linking is effected either chemically, by recombinant expression of a fusion protein in instances when the targeted agent is a protein, and by combinations of chemical and recombinant expression. The targeting agent is one that will preferentially bind to a selected tissue or cell type, such as a tumor cell surface antigen or other tissue specific antigen.

Methods for preparing conjugates are known to those of skill in the art. For example, aequorin that is designed for conjugation and conjugates containing such aequorin have been produced [see, e.g., International PCT application No. WO 94/18342; see, also Smith et al. (1995) in American Biotechnology Laboratory]. Aequorin has been conjugated to an antibody molecule by means of a sulfhydryl-reacting binding agent (Stultz et al. (1992) Use of Recombinant Biotinylated Apoaequorin from *Escherichia coli*. Biochemistry 31, 1433-1442). Such methods may be adapted for use herein to produce aequorin coupled to protein or other such molecules, which are useful

-116-

as targeting agents. *Vargula* luciferase has also been linked to other molecules [see, e.g., Japanese application No. JP 5064583, March 19, 1993]. Such methods may be adapted for use herein to produce aequorin coupled to protein or other such molecules, which are useful as targeting agents.

5 Aequorin-antibody conjugates have been employed to detect the presence of or quantitate a particular antigen in a biological sample by direct correlation to the light emitted from the bioluminescent reaction.

 As an alternative, the *Renilla* GFP or *Renilla mulleri* or *Gaussia* luciferase or a component of the bioluminescence generating system may be modified for
10 linkage, such as by addition of amino acid residues that are particularly suitable for linkage to the selected substrate. This can be readily effected by modifying the DNA and expressing such modified DNA to produce luciferase with additional residues at the N- or C-terminus.

 Selection of the system depends upon factors such as the desired color
15 and duration of the bioluminescence desired as well as the particular item. Selection of the targeting agent primarily depends upon the type and characteristics of neoplasia or tissue to be visualized and the setting in which visualization will be performed.

20 c. **Practice of the reactions in combination with targeting agents**

 The particular manner in which each bioluminescence system will be combined with a selected targeting agent will be a function of the agent and the neoplasia or tissue to be visualized. In general, however, a luciferin, *Renilla* GFP, *Renilla mulleri*, *Pleuromamma* or *Gaussia* luciferase or other luciferase, of
25 the reaction will be conjugated to the targeting agent, administered to an animal prior to surgery. During the surgery, the tissues of interest are contacted with the remaining component(s) of a bioluminescence generating system. Any tissue to which or with which the targeting agent reacts will glow.

-117-

Any color of visible light produced by a bioluminescence generating system is contemplated for use in the methods herein. Preferably the visible light is a combination of blue, green and/or red light of varying intensities and wavelengths. For visualizing neoplasia or specialty tissues through mammalian
5 tissues or tumors deeply embedded in tissue, longer wavelengths of visible light, i.e., red and near infrared light, is preferred because wavelengths of near infrared light of about 700-1300 nm are known to penetrate soft tissue and bone [e.g., see U.S. Patent No. 4,281,645].

In other embodiments, the conjugate can be applied to the tissues during
10 surgery, such as by spraying a sterile solution over the tissues, followed by application of the remaining components. Tissues that express the targeted antigen will glow.

The reagents may be provided in compositions, such as suspensions, as powders, as pastes or any in other suitable sterile form. They may be provided
15 as sprays, aerosols, or in any suitable form. The reagents may be linked to a matrix, particularly microbeads suitable for in vivo use and of size that they pass through capillaries. Typically all but one or more, though preferably all but one, of the components necessary for the reaction will be mixed and provided together; reaction will be triggered contacting the mixed component(s) with the
20 remaining component(s), such as by adding Ca^{2+} , FMN with reductase, FMNH_2 , ATP, air or oxygen.

In preferred embodiments the luciferase or luciferase/luciferin will be provided in combination with the targeting agent before administration to the patient. The targeting agent conjugate will then be contacted in vivo with the
25 remaining components. As will become apparent herein, there are a multitude of ways in which each system may be combined with a selected targeting agent.

J. COMBINATIONS

In addition, the above-described *Pleuromamma*, *Gaussia* or *Renilla*
30 luciferases and/or *Renilla* and *Ptilosarcus* GFPs can be used in combination with articles of manufacture to produce novelty items. Such items and methods for preparation are described in detail in copending U.S. application Serial Nos.

-118-

08/597,274 and 08/757,046. The luciferases and/or GFPs provided herein may be used in the methods and items as provided in the copending applications.

These novelty items, which are articles of manufacture, are designed for entertainment, recreation and amusement, and include, but are not limited to:

- 5 toys, particularly squirt guns, toy cigarettes, toy "Halloween" eggs, footbags and board/card games; finger paints and other paints, slimy play material; textiles, particularly clothing, such as shirts, hats and sports gear suits, threads and yarns; bubbles in bubble making toys and other toys that produce bubbles; balloons; figurines; personal items, such as bath powders, body lotions, gels,
- 10 powders and creams, nail polishes, cosmetics including make-up, toothpastes and other dentifrices, soaps, body paints, and bubble bath; items such as inks, paper; foods, such as gelatins, icings and frostings; fish food containing luciferins and transgenic fish, particularly transgenic fish that express a luciferase; plant food containing a luciferin or luciferase, preferably a luciferin for
- 15 use with transgenic plants that express luciferase; and beverages, such as beer, wine, champagne, soft drinks, and ice cubes and ice in other configurations; fountains, including liquid "fireworks" and other such jets or sprays or aerosols of compositions that are solutions, mixtures, suspensions, powders, pastes, particles or other suitable form.

- 20 Any article of manufacture that can be combined with a bioluminescence-generating system as provided herein and thereby provide entertainment, recreation and/or amusement, including use of the items for recreation or to attract attention, such as for advertising goods and/or services that are associated with a logo or trademark is contemplated herein. Such uses
- 25 may be in addition to or in conjunction with or in place of the ordinary or normal use of such items. As a result of the combination, the items glow or produce, such as in the case of squirt guns and fountains, a glowing fluid or spray of liquid or particles. K. **METHODS OF USE**

1. Methods for diagnosis of neoplasms and other tissues

- 30 Methods for diagnosis and visualization of tissues *in vivo* or *in situ*, preferably neoplastic tissue, using compositions containing a *Renilla mulleri* or *Ptilosarcus* GFP and/or a *Renilla mulleri*, *Pleuromamma* or *Gaussia* luciferase are

-119-

provided. For example, the *Renilla mulleri* GFP protein can be used in conjunction with diagnostic systems that rely on bioluminescence for visualizing tissues *in situ*, such as those described in co-pending application Serial No. 08/908,909. The systems are particularly useful for visualizing and detecting

5 neoplastic tissue and specialty tissue, such as during non-invasive and invasive procedures. The systems include compositions containing conjugates that include a tissue specific, particularly a tumor-specific, targeting agent linked to a targeted agent, such as a *Renilla mulleri* GFP, a luciferase or luciferin. The systems also include a second composition that contains the remaining

10 components of a bioluminescence generating reaction and/or the GFP. In some embodiments, all components, except for activators, which are provided *in situ* or are present in the body or tissue, are included in a single composition.

In particular, the diagnostic systems include two compositions. A first composition that contains conjugates that, in preferred embodiments, include

15 antibodies directed against tumor antigens conjugated to a component of the bioluminescence generating reaction, a luciferase or luciferin, preferably a luciferase are provided. In certain embodiments, conjugates containing tumor-specific targeting agents are linked to luciferases or luciferins. In other

embodiments, tumor-specific targeting agents are linked to microcarriers that

20 are coupled with, preferably more than one of the bioluminescence generating components, preferably more than one luciferase molecule.

The second composition contains the remaining components of a bioluminescence generating system, typically the luciferin or luciferase substrate. In some embodiments, these components, particularly the luciferin

25 are linked to a protein, such as a serum albumin, or other protein carrier. The carrier and time release formulations, permit systemically administered components to travel to the targeted tissue without interaction with blood cell components, such as hemoglobin that deactivates the luciferin or luciferase.

-120-

2. Methods of diagnosing diseases

Methods for diagnosing diseases, particularly infectious diseases, using chip methodology, a luciferase/luciferin bioluminescence-generating system, including a *Gaussia*, *Pleuromamma* or *Renilla mulleri* luciferase and/or a
5 *Ptilosarcus* or *Renilla mulleri* GFP, are provided. In particular, the chip includes an integrated photodetector that detects the photons emitted by the bioluminescence-generating system and/or GFP.

In one embodiment, the chip is made using an integrated circuit with an array, such as an X-Y array, of photodetectors, such as that described in co-
10 pending U.S. application Serial No. 08/990,103. The surface of circuit is treated to render it inert to conditions of the diagnostic assays for which the chip is intended, and is adapted, such as by derivatization for linking molecules, such as antibodies. A selected antibody or panel of antibodies, such as an antibody specific for particularly bacterial antigen, is affixed to the surface of
15 the chip above each photodetector. After contacting the chip with a test sample, the chip is contacted with a second antibody linked to the GFP, such as the *Renilla* GFP, to form a chimeric antibody- GFP fusion protein or an antibody linked to a component of a bioluminescence generating system, such as a *Pleuromamma*, *Gaussia* or *R. mulleri* luciferase. The antibody is specific for the
20 antigen. The remaining components of the bioluminescence generating reaction are added, and, if any of the antibodies linked to a component of a bioluminescence generating system are present on the chip, light will be generated and detected by the adjacent photodetector. The photodetector is operatively linked to a computer, which is programmed with information
25 identifying the linked antibodies, records the event, and thereby identifies antigens present in the test sample.

3. Methods for generating chimeric *Renilla* or *Ptilosarcus* GFP, *Renilla mulleri* luciferase, *Pleuromamma* luciferase and *Gaussia* luciferase fusion proteins

30 Methods for generating chimeric GFP and luciferase fusion proteins are provided. The methods include linking DNA encoding a gene of interest, or portion thereof, to DNA encoding a GFP or luciferase provided herein in the

-121-

same translational reading frame. The encoded-protein of interest may be linked in-frame to the amino- or carboxyl-terminus of the GFP or luciferase. The DNA encoding the chimeric protein is then linked in operable association with a promoter element of a suitable expression vector. Alternatively, the promoter element can be obtained directly from the targeted gene of interest and the promoter-containing fragment linked upstream from the GFP or luciferase coding sequence to produce chimeric GFP proteins.

For example, a chimeric fusion containing the *Gaussia* luciferase encoding DNA linked to the N-terminal portion of a cellulose binding domain is provided (see, SEQ ID Nos. 21 and 22).

4. Cell-based assays for identifying compounds

Methods for identifying compounds using recombinant cells that express heterologous DNA encoding a *Renilla mulleri* or *Ptilosarcus* GFP under the control of a promoter element of a gene of interest are provided. The recombinant cells can be used to identify compounds or ligands that modulate the level of transcription from the promoter of interest by measuring GFP-mediated fluorescence. Recombinant cells expressing chimeric GFPs may also be used for monitoring gene expression or protein trafficking, or determining the cellular localization of the target protein by identifying localized regions of GFP-mediated fluorescence within the recombinant cell.

L. KITS

Kits may be prepared containing the *Gaussia*, *Pleuromamma* or *Renilla mulleri* luciferase or the *Renilla* and *Ptilosarcus* GFPs for use in diagnostic and immunoassay methods and with the novelty items, including those described herein.

In one embodiment, the kits contain appropriate reagents and an article of manufacture for generating bioluminescence in combination with the article. These kits, for example, can be used with a bubble-blowing or producing toy or with a squirt gun. These kits can also include a reloading or charging cartridge.

In another embodiment, the kits are used for detecting and visualizing neoplastic tissue and other tissues and include a first composition that contains the luciferase and/or *Renilla mulleri* or *Ptilosarcus* GFP and at least one

-122-

component of a bioluminescence generating system, and a second that contains the activating composition, which contains the remaining components of the bioluminescence generating system and any necessary activating agents.

In other embodiments, the kits are used for detecting and identifying
5 diseases, particularly infectious diseases, using multi-well assay devices and include a multi-well assay device containing a plurality of wells, each having an integrated photodetector, to which an antibody or panel of antibodies specific for one or more infectious agents are attached, and composition containing a secondary antibody, such as an antibody specific for the infectious agent that is
10 linked, for example, to a *Renilla mulleri* GFP protein, a chimeric antibody-*Renilla mulleri* GFP fusion protein, F(Ab)₂ antibody fragment-*Renilla mulleri* GFP fusion protein or to such conjugates containing the, for example, *Gaussia* or *Renilla mulleri*, luciferase. A second composition containing the remaining components of a bioluminescence generating system, such as system that emits a
15 wavelength of light within the excitation range of the GFP, such as species of *Renilla* or *Aequorea*, for exciting the *Renilla mulleri* luciferase, which produces green light that is detected by the photodetector of the device to indicate the presence of the agent.

In further embodiments, the kits contain the components of the
20 diagnostic systems. The kits comprise compositions containing the conjugates, preferably *Renilla* or *Ptilosarcus* GFP or *Gaussia*, or *Pleuromamma* or *Renilla mulleri* luciferase and remaining bioluminescence generating system components. The first composition in the kit typically contains the targeting agent conjugated to a GFP or luciferase. The second composition, contains at
25 least the luciferin (substrate) and/or luciferase. Both compositions are formulated for systemic, local or topical application to a mammal. In alternative embodiments, the first composition contains the luciferin linked to a targeting agent, and the second composition contains the luciferase or the luciferase and a GFP.

30 In general, the packaging is non-reactive with the compositions contained therein and where needed should exclude water and or air to the degree those substances are required for the luminescent reaction to proceed.

-123-

Diagnostic applications may require specific packaging. The bioluminescence generating reagents may be provided in pellets, encapsulated as micro or macro-capsules, linked to matrices, preferably biocompatible, more preferably biodegradable matrices, and included in or on articles of manufacture, or as mixtures in chambers within an article of manufacture or in some other configuration. For example, a composition containing luciferase conjugate will be provided separately from, and for use with, a separate composition containing a bioluminescence substrate and bioluminescence activator.

Similarly, the *Renilla* or *Ptilosarcus* GFP, *Pleuromamma*, *Renilla mulleri* or *Gaussia* luciferase or luciferin may be provided in a composition that is a mixture, suspension, solution, powder, paste or other suitable composition separately from or in combination with the remaining components, but in the absence of an activating component. Upon contacting the conjugate, which has been targeted to a selected tissue, with this composition the reaction commences and the tissue glows. In preferred embodiments, the tissue glows green emitting light near 510 nm. The luciferase, GFP and bioluminescence substrate, for example, are packaged to exclude water and/or air, the bioluminescence activator. Upon administration and release at the targeted site, the reaction with salts or other components at the site, including air in the case of surgical procedures, will activate the components.

1. Dispensing and Packaging Apparatus for Combination with the GFP and Bioluminescent System Components

The bioluminescence systems, described in detail herein, include at least three components: a bioluminescence substrate [e.g., a luciferin], a luciferase [e.g., a luciferase or photoprotein], preferably *Gaussia*, *Pleuromamma* or *Renilla mulleri* luciferase, and a bioluminescence activator or activators [e.g., molecular oxygen or Ca^{2+}], and optionally a *Renilla* or *Ptilosarcus* GFP. The dispensing and packaging apparatus are configured to keep at least one of the components separate from the remaining components, until generation of bioluminescence is desired. Detailed descriptions of such apparatus are described in copending, commonly owned U.S. application Serial Nos. 08/757,046 and 08/597,274, which are incorporated by reference herein.

-124-

2. Capsules, pellets, liposomes, endosomes, vacuoles, micronized particles

In certain embodiments sequestering of the components of one of the compositions from the environment prior to use or provision of the components in particulate form, such as microparticles, may be necessary. Examples of suitable means for such use include encapsulating bioluminescent generating system components in one or micro- [up to about 100 μm in size] or macroparticles [larger than 100 μm] of material that permits release of the contents, such as by diffusion or by dissolution of the encapsulating material. Microparticles to which a plurality of conjugates can be linked are among the preferred embodiments. The microparticles are biocompatible and preferably of a size that can pass through capillary walls.

Liposomes and other encapsulating vehicles [see, e.g., U.S. Patent No. 4,525,306, which describes encapsulation of compounds in gelatin; U.S. Patent Nos. 4,021,364, 4,225,581, 4,269,821, 4,322,311, 4,324,683, 4,329,332, 4,525,306, 4,963,368 describe encapsulation of biologically active materials in various polymers] known to those of skill in the art, including those discussed herein and known to those of skill in the art [such as soluble paper, see U.S. Patent No. 3,859,125].

20 a. Encapsulating vehicles in general

The components of the bioluminescence generating system, except for the oxygen or water or Ca^{2+} , depending upon the selected system can be incorporated into encapsulating material, such as liposomes, that protect the contents from the environment until placed into conditions that cause release of the contents into the environment. Encapsulating material contemplated for use herein includes liposomes and other such materials used for encapsulating chemicals, such as drug delivery vehicles.

b. Encapsulating vehicles -liposomes

For example, liposomes that dissolve and slowly release the components into the medium, such as the blood, which contains dissolved oxygen or Ca^{2+} or even ATP for the luciferase system are contemplated herein. They can be formulated in compositions, such as solutions, suspensions, gels, lotions,

-125-

creams, and ointments, for topical application, such as procedures for diagnosing or visualizing melanomas. Liposomes and other slow release encapsulating compositions are well known and can be adapted for use in for slow release delivery of bioluminescence generating components. Typically the

5 GFP, luciferin and/or luciferase will be encapsulated in the absence of oxygen or Ca^{2+} or ATP or other activating component. Upon release into the environment or medium containing this component at a suitable concentration, the reaction will proceed and a glow will be produced. Generally the concentrations of encapsulated components should be relatively high, perhaps 0.1 - 1 mg/ml or

10 more, to ensure high enough local concentrations upon release to be visible.

Liposomes or other sustained release delivery system that are formulated in an ointment or sustained release topical vehicle, for example, would be suitable for use in a body paint, lotion. Those formulated as a suspension would be useful as a spray. Numerous ointments and suitable liposome

15 formulations are known [see, e.g., Liposome Technology, Targeted Drug Delivery and Biological Interaction, vol. III, G. Gregoriadis ed., CRC Press, Inc., 1984; U.S. Patent Nos. 5,470,881; 5,366,881; 5,296,231; 5,272,079; 5,225,212; 5,190,762; 5,188,837; 5,188,837; 4,921,757; 4,522,811]. For example, an appropriate ointment vehicle would contain petrolatum, mineral oil

20 and/or anhydrous liquid lanolin. Sustained release vehicles such as liposomes, membrane or contact lens delivery systems, or gel-forming plastic polymers would also be suitable delivery vehicles. Liposomes for topical delivery are well known [see, e.g., U.S. Patent No. 5,296,231; Mezei *et al.* (1980) "Liposomes -A selective drug delivery system for the topical route of administration, I. lotion

25 dosage form" *Life Sciences* 26:1473-1477; Mezei *et al.* (1981) "Liposomes -A selective drug delivery system for the topical route of administration: gel dosage form" *Journal of Pharmacy and Pharmacology* 34:473-474; Gesztes *et al.* (1988) "Topical anaesthesia of the skin by liposome -encapsulated tetracaine" *Anesthesia and Analgesia* 67:1079-1081; Patel (1985) "Liposomes as a

30 controlled-release system", *Biochemical Soc. Trans.* 13:513-516; Wohlrab *et al.* (1987) "Penetration kinetics of liposomal hydrocortisone in human skin" *Dermatologica* 174:18-22].

-126-

Liposomes are microcapsules [diameters typically on the order of less than 0.1 to 20 μm] that contain selected mixtures and can slowly release their contents in a sustained release fashion. Targeted liposomes or other capsule, particularly a time release coating, that dissolve upon exposure to oxygen, air, moisture, visible or ultraviolet [UV] light or a particular pH or temperature [see, e.g., U.S. Patent No. 4,882,165; Kusumi *et al.* (1989) *Chem. Lett.* no.3 433-436; Koch Troels *et al.* (1990) *Bioconjugate Chem.* 4:296-304; U.S. Patent No. 5,482,719; U.S. Patent No. 5,411,730; U.S. Patent No. 4,891,043; Straubinger *et al.* (1983) *Cell* 32:1069-1079; and Straubinger *et al.* (1985) *FEBS Lttrs.* 179:148-154; and Duzgunes *et al.* in Chapter 11 of the book CELL FUSION, edited by A. E. Sowers; Ellens *et al.* (1984) *Biochemistry* 23:1532-1538; Yatvin *et al.* (1987) *Methods in Enzymology* 149:77-87] may be used. Liposome formulations for use in baking [see, e.g., U.S. Patent No. 4,999,208] are available. They release their contents when eaten or heated. Such liposomes may be suitable for intravenous or local administration.

Liposomes be prepared by methods known to those of skill in the art [see, e.g., Kimm *et al.* (1983) *Bioch. Bioph. Acta* 728:339-398; Assil *et al.* (1987) *Arch Ophthalmol.* 105:400; and U.S. Patent No. 4,522,811, and other citations herein and known to those of skill in the art].

Liposomes that are sensitive to low pH [see, e.g., U.S. Patent No. 5,352,448, 5,296,231; 5,283,122; 5,277,913, 4,789,633] are particularly suitable for use with alkaline agents. Upon contact with the low pH detergent or soap composition or a high pH composition, the contents of the liposome will be released. Other components, particularly Ca^+ or the presence of dissolved O_2 in the water will cause the components to glow as they are released. Temperature sensitive liposomes are also suitable for use in bath powders for release into the warm bath water.

c. Encapsulating vehicles -gelatin and polymeric vehicles

Macro or microcapsules made of gelatin or other such polymer that dissolve or release their contents on contact with air or light or changes in temperature may also be used to encapsulate components of the bioluminescence generating systems.

-127-

Such microcapsules or macrocapsules may also be conjugated to a targeting agent, e.g., an antibody, such that the GFP or luciferase and bioluminescence generating components are delivered to the target by the antibody and then the components are released to produce a glow.

5 The aequorin system is particularly suitable for this application. It can be encapsulated in suspension or solution or as a paste, or other suitable form, of buffer with sufficient chelating agent, such as EDTA, to prevent discharge of the bioluminescence. Upon exposure of the capsule [microcapsule or
10 microcapsule] to moisture that contains Ca^{2+} , such as in a buffer or blood, the released components will glow.

Thus, encapsulated bioluminescence generating components can be used in combination with a variety of targeting agents and thereby release the luciferase/luciferin, such as the *Renilla mulleri*, *Pleuromamma*, *Ptilosarcus* or *Gaussia* system, which will light upon exposure to air.

15 Other encapsulating containers or vehicles for use with the bioluminescence systems are those that dissolve sufficiently in water to release their contents, or that are readily opened when squeezed in the hand or from which the contents diffuse when mixed with a aqueous mixture. These containers can be made to exclude water, so that the bioluminescent system
20 components may be desiccated and placed therein. Upon exposure to water, such as in an aqueous composition solution or in the atmosphere, the vehicle dissolves or otherwise releases the contents, and the components react and glow. Similarly, some portion less than all of the bioluminescence generating components may themselves be prepared in pellet form. For example, the
25 component(s) may be mixed with gelatin or similar hardening agent, poured into a mold, if necessary and dried to a hard, water soluble pellet. The encapsulating containers or vehicles may be formed from gelatin or similar water soluble material that is biocompatible.

d. Endosomes and vacuoles

30 Vehicles may be produced using endosomes or vacuoles from recombinant host cells in which the *Renilla* or *Ptilosarcus* GFPs or *Renilla mulleri*, *Pleuromamma* or *Gaussia* luciferase is expressed using method known to those

-128-

of skill in the art [see, e.g., U.S. Patent Nos. 5,284,646, 5,342,607, 5,352,432, 5,484,589, 5,192,679, 5,206,161, and 5,360,726]. For example, aequorin that is produced by expression in a host, such as E. coli, can be isolated within vesicles, such as endosomes or vacuoles, after protein synthesis. Using routine methods the cells are lysed and the vesicles are released with their contents intact. The vesicles will serve as delivery vehicles. When used they will be charged with a luciferin, such as a coelenterazine, and dissolved oxygen, such as by diffusion, under pressure, or other appropriate means.

10 e. **Micronized particles**

The bioluminescence generating system components that are suitable for lyophilization, such as the aequorin photoprotein, the *Renilla* system, *Ptilosarcus*, *Pleuromamma* and the *Gaussia* systems, can be micronized to form fine powder and stored under desiccating conditions, such as with a desiccant.

15 Contact with dissolved oxygen or Ca^{2+} in the air or in a mist that can be supplied or in added solution will cause the particles to dissolve and glow.

3. **Immobilized systems**

a. **Matrix materials**

20 In some embodiments, it will be desirable to provide at least the GFPs or one component of the bioluminescence generating system linked to a matrix substrate, which can then be locally or systemically administered. The matrix substrate will be biocompatible. When desired, a mixture or mixtures(s) containing the remaining components, typically a liquid mixture is applied, as by pouring or spraying onto the matrix substrate, to produce a glow. For example, 25 the aequorin photoprotein, including coelenterazine and oxygen, is linked to the substrate. When desired a liquid containing Ca^{2+} , such as tap water or, preferably, a liquid mixture containing the Ca^{2+} in an appropriate buffer, is contacted, such as by spraying, with the matrix with linked luciferase. Upon contacting in the presence of a GFP the material glows green.

30 In other embodiments, the *Renilla* GFP, *Renilla mulleri* or *Gaussia*, *Pleuromamma* luciferase or other luciferase, such as a *Vargula* luciferase, is linked to the substrate material, and contacted with a liquid mixture containing

-129-

the luciferin in an appropriate buffer. Contacting can be effected by spraying or pouring or other suitable manner. The matrix material is incorporated into, onto or is formed into an article of manufacture, such as surgical sponge or as part of a microbead.

- 5 It is understood that the precise components and optimal means for application or storage are a function of the selected bioluminescence system. The concentrations of the components, which can be determined empirically, are not critical, but must be sufficient to produce a visible glow when combined. Typical concentrations are as low as nanomoles/l, preferably on the
- 10 order of mg/l or higher. The concentration on the substrate is that produced when a composition containing such typical concentration is applied to the material. Again, such ideal concentrations can be readily determined empirically by applying the first composition, letting it dry, spraying the second composition, and observing the result.
- 15 The matrix material substrates contemplated herein are generally insoluble materials used to immobilize ligands and other molecules, and are those that used in many chemical syntheses and separations. Such matrices are fabricated preferably from biocompatible, more preferably from biodegradable materials. Such substrates, also called matrices, are used, for
- 20 example, in affinity chromatography, in the immobilization of biologically active materials, and during chemical syntheses of biomolecules, including proteins, amino acids and other organic molecules and polymers. The preparation of and use of matrices is well known to those of skill in this art; there are many such materials and preparations thereof known. For example, naturally-occurring
- 25 matrix materials, such as agarose and cellulose, may be isolated from their respective sources, and processed according to known protocols, and synthetic materials may be prepared in accord with known protocols. Other matrices for use herein may comprise proteins, for example carrier molecules, such as albumin.

-130-

The substrate matrices are typically insoluble materials that are solid, porous, deformable, or hard, and have any required structure and geometry, including, but not limited to: beads, pellets, disks, capillaries, hollow fibers, needles, solid fibers, random shapes, thin films and membranes. Thus, the item
5 may be fabricated from the matrix material or combined with it, such by coating all or part of the surface or impregnating particles.

Typically, when the matrix is particulate, the particles are at least about 10-2000 μM , but may be smaller or larger, depending upon the selected application. Selection of the matrices will be governed, at least in part, by their
10 physical and chemical properties, such as solubility, functional groups, mechanical stability, surface area swelling propensity, hydrophobic or hydrophilic properties and intended use. For use herein, the matrices are preferably biocompatible, more preferably biodegradable matrices.

If necessary the support matrix material can be treated to contain an
15 appropriate reactive moiety or in some cases the may be obtained commercially already containing the reactive moiety, and may thereby serve as the matrix support upon which molecules are linked. Materials containing reactive surface moieties such as amino silane linkages, hydroxyl linkages or carboxysilane linkages may be produced by well established surface chemistry techniques
20 involving silanization reactions, or the like. Examples of these materials are those having surface silicon oxide moieties, covalently linked to gamma-amino-propylsilane, and other organic moieties; N-[3-(triethoxysilyl)propyl]phthelamic acid; and bis-(2-hydroxyethyl)aminopropyltriethoxysilane. Exemplary of readily available materials containing amino group reactive functionalities, include, but
25 are not limited to, para-aminophenyltriethoxysilane. Also derivatized polystyrenes and other such polymers are well known and readily available to those of skill in this art [e.g., the Tentagel[®] Resins are available with a multitude of functional groups, and are sold by Rapp Polymere, Tubingen, Germany; see, U.S. Patent No. 4,908,405 and U.S. Patent No. 5,292,814; see, also Butz *et al.*
30 (1994) *Peptide Res.* 7:20-23; Kleine *et al.* (1994) *Immunobiol.* 190:53-66].

These matrix materials include any material that can act as a support matrix for attachment of the molecules of interest. Such materials are known

-131-

to those of skill in this art, and include those that are used as a support matrix. These materials include, but are not limited to, inorganic, natural polymers, and synthetic polymers, including, but are not limited to: cellulose, cellulose derivatives, acrylic resins, glass, silica gels, polystyrene, gelatin, polyvinyl pyrrolidone, co-polymers of vinyl and acrylamide, polystyrene cross-linked with divinylbenzene or the like [see, Merrifield (1964) *Biochemistry* 3:1385-1390], polyacrylamides, latex gels, polystyrene, dextran, polyacrylamides, rubber, silicon, plastics, nitrocellulose, celluloses, natural sponges. Of particular interest herein, are highly porous glasses [see, e.g., U.S. Patent No. 4,244,721] and others prepared by mixing a borosilicate, alcohol and water.

Synthetic matrices include, but are not limited to: acrylamides, dextran-derivatives and dextran co-polymers, agarose-polyacrylamide blends, other polymers and co-polymers with various functional groups, methacrylate derivatives and co-polymers, polystyrene and polystyrene copolymers [see, e.g., Merrifield (1964) *Biochemistry* 3:1385-1390; Berg et al. (1990) in *Innovation Perspect. Solid Phase Synth. Collect. Pap., Int. Symp., 1st*, Epton, Roger (Ed), pp. 453-459; Berg et al. (1989) in *Pept., Proc. Eur. Pept. Symp., 20th*, Jung, G. et al. (Eds), pp. 196-198; Berg et al. (1989) *J. Am. Chem. Soc.* 111:8024-8026; Kent et al. (1979) *Isr. J. Chem.* 17:243-247; Kent et al. (1978) *J. Org. Chem.* 43:2845-2852; Mitchell et al. (1976) *Tetrahedron Lett.* 42:3795-3798; U.S. Patent No. 4,507,230; U.S. Patent No. 4,006,117; and U.S. Patent No. 5,389,449]. Methods for preparation of such matrices are well-known to those of skill in this art.

Synthetic matrices include those made from polymers and co-polymers such as polyvinylalcohols, acrylates and acrylic acids such as polyethylene-co-acrylic acid, polyethylene-co-methacrylic acid, polyethylene-co-ethylacrylate, polyethylene-co-methyl acrylate, polypropylene-co-acrylic acid, polypropylene-co-methyl-acrylic acid, polypropylene-co-ethylacrylate, polypropylene-co-methyl acrylate, polyethylene-co-vinyl acetate, polypropylene-co-vinyl acetate, and those containing acid anhydride groups such as polyethylene-co-maleic anhydride, polypropylene-co-maleic anhydride and the

-132-

like. Liposomes have also been used as solid supports for affinity purifications [Powell *et al.* (1989) *Biotechnol. Bioeng.* 33:173].

- For example, U.S. Patent No. 5,403,750, describes the preparation of polyurethane-based polymers. U.S. Pat. No. 4,241,537 describes a plant
- 5 growth medium containing a hydrophilic polyurethane gel composition prepared from chain-extended polyols; random copolymerization is preferred with up to 50% propylene oxide units so that the prepolymer will be a liquid at room temperature. U.S. Pat. No. 3,939,123 describes lightly crosslinked polyurethane polymers of isocyanate terminated prepolymers containing poly(ethyleneoxy)
- 10 glycols with up to 35% of a poly(propyleneoxy) glycol or a poly(butyleneoxy) glycol. In producing these polymers, an organic polyamine is used as a crosslinking agent. Other matrices and preparation thereof are described in U.S. Patent Nos. 4,177,038, 4,175,183, 4,439,585, 4,485,227, 4,569,981, 5,092,992, 5,334,640, 5,328,603
- 15 U.S. Patent No. 4,162,355 describes a polymer suitable for use in affinity chromatography, which is a polymer of an aminimide and a vinyl compound having at least one pendant halo-methyl group. An amine ligand, which affords sites for binding in affinity chromatography is coupled to the polymer by reaction with a portion of the pendant halo-methyl groups and the
- 20 remainder of the pendant halo-methyl groups are reacted with an amine containing a pendant hydrophilic group. A method of coating a substrate with this polymer is also described. An exemplary aminimide is 1,1-dimethyl-1-(2-hydroxyoctyl)amine methacrylimide and vinyl compound is a chloromethyl styrene.
- 25 U.S. Patent No. 4,171,412 describes specific matrices based on hydrophilic polymeric gels, preferably of a macroporous character, which carry covalently bonded D-amino acids or peptides that contain D-amino acid units. The basic support is prepared by copolymerization of hydroxyalkyl esters or hydroxyalkylamides of acrylic and methacrylic acid with crosslinking acrylate or
- 30 methacrylate comonomers are modified by the reaction with diamines, aminoacids or dicarboxylic acids and the resulting carboxyterminal or aminoterminal groups are condensed with D-analogs of aminoacids or peptides.

-133-

The peptide containing D-aminoacids also can be synthesized stepwise on the surface of the carrier.

U.S. Patent No. 4,178,439 describes a cationic ion exchanger and a method for preparation thereof. U.S. Patent No. 4,180,524 describes chemical
5 syntheses on a silica support.

Immobilized Artificial Membranes [IAMs; see, e.g., U.S. Patent Nos. 4,931,498 and 4,927,879] may also be used. IAMs mimic cell membrane environments and may be used to bind molecules that preferentially associate with cell membranes [see, e.g., Pidgeon et al. (1990) Enzyme Microb. Technol.
10 12:149].

These materials are also used for preparing articles of manufacture, surgical sponges soaps, and other items, and thus are amenable to linkage of molecules, either the luciferase, luciferin, mixtures of both. For example, matrix particles may be impregnated into items that will then be
15 contacted with an activator.

Kits containing the item including the matrix material with or without the coating of the GFPs or bioluminescence generating components, and compositions containing the remaining components are provided.

b. Immobilization and activation

20 Numerous methods have been developed for the immobilization of proteins and other biomolecules onto insoluble or liquid supports [see, e.g., Mosbach (1976) Methods in Enzymology 44; Weetall (1975) Immobilized Enzymes, Antigens, Antibodies, and Peptides; and Kennedy et al. (1983) Solid Phase Biochemistry, Analytical and Synthetic Aspects, Scouten, ed., pp.
25 253-391; see, generally, Affinity Techniques. Enzyme Purification: Part B. Methods in Enzymology, Vol. 34, ed. W. B. Jakoby, M. Wilchek, Acad. Press, N.Y. (1974); Immobilized Biochemicals and Affinity Chromatography, Advances in Experimental Medicine and Biology, vol. 42, ed. R. Dunlap, Plenum Press, N.Y. (1974)].

30 Among the most commonly used methods are absorption and adsorption or covalent binding to the support, either directly or via a linker, such as the numerous disulfide linkages, thioether bonds, hindered disulfide bonds, and

-134-

covalent bonds between free reactive groups, such as amine and thiol groups, known to those of skill in art [see, e.g., the PIERCE CATALOG, ImmunoTechnology Catalog & Handbook, 1992-1993, which describes the preparation of and use of such reagents and provides a commercial source for
5 such reagents; and Wong (1993) Chemistry of Protein Conjugation and Cross Linking, CRC Press; see, also DeWitt et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90:6909; Zuckermann et al. (1992) J. Am. Chem. Soc. 114:10646; Kurth et al. (1994) J. Am. Chem. Soc. 116:2661; Ellman et al. (1994) Proc. Natl. Acad. Sci. U.S.A. 91:4708; Sucholeiki (1994) Tetrahedron Lttrs. 35:7307; and Su-
10 Sun Wang (1976) J. Org. Chem. 41:3258; Padwa et al. (1971) J. Org. Chem. 41:3550 and Vedejs et al. (1984) J. Org. Chem. 49:575, which describe photosensitive linkers]

To effect immobilization, a solution of the protein or other biomolecule is contacted with a support material such as alumina, carbon, an ion-exchange
15 resin, cellulose, glass or a ceramic. Fluorocarbon polymers have been used as supports to which biomolecules have been attached by adsorption [see, U.S. Pat. No. 3,843,443; Published International PCT Application WO/86 03840]. For purposes herein, the support material will be biocompatible (i.e., suitable for use in the body).

20 A large variety of methods are known for attaching biological molecules, including proteins and nucleic acids, molecules to solid supports [see, e.g., U.S. Patent No. 5451683]. For example, U.S. Pat. No. 4,681,870 describes a method for introducing free amino or carboxyl groups onto a silica matrix. These groups may subsequently be covalently linked to other groups, such as a
25 protein or other anti-ligand, in the presence of a carbodiimide. Alternatively, a silica matrix may be activated by treatment with a cyanogen halide under alkaline conditions. The anti-ligand is covalently attached to the surface upon addition to the activated surface. Another method involves modification of a polymer surface through the successive application of multiple layers of biotin,
30 avidin and extenders [see, e.g., U.S. Patent No. 4,282,287]; other methods involve photoactivation in which a polypeptide chain is attached to a solid substrate by incorporating a light-sensitive unnatural amino acid group into the

-135-

polypeptide chain and exposing the product to low-energy ultraviolet light [see, e.g., U.S. Patent No. 4,762,881]. Oligonucleotides have also been attached using a photochemically active reagents, such as a psoralen compound, and a coupling agent, which attaches the photoreagent to the substrate [see, e.g.,
5 U.S. Patent No. 4,542,102 and U.S. Patent No. 4,562,157]. Photoactivation of the photoreagent binds a nucleic acid molecule to the substrate to give a surface-bound probe.

Covalent binding of the protein or other biomolecule or organic molecule or biological particle to chemically activated solid matrix supports such as glass,
10 synthetic polymers, and cross-linked polysaccharides is a more frequently used immobilization technique. The molecule or biological particle may be directly linked to the matrix support or linked via linker, such as a metal [see, e.g., U.S. Patent No. 4,179,402; and Smith et al. (1992) Methods: A Companion to Methods in Enz. 4:73-78]. An example of this method is the cyanogen bromide
15 activation of polysaccharide supports, such as agarose. The use of perfluorocarbon polymer-based supports for enzyme immobilization and affinity chromatography is described in U.S. Pat. No. 4,885,250]. In this method the biomolecule is first modified by reaction with a perfluoroalkylating agent such as perfluorooctylpropylisocyanate described in U.S. Pat. No. 4,954,444. Then, the
20 modified protein is adsorbed onto the fluorocarbon support to effect immobilization.

The activation and use of matrices are well known and may be effected by any such known methods [see, e.g., Hermanson et al. (1992) Immobilized Affinity Ligand Techniques, Academic Press, Inc., San Diego]. For example, the
25 coupling of the amino acids may be accomplished by techniques familiar to those in the art and provided, for example, in Stewart and Young, 1984, Solid Phase Synthesis, Second Edition, Pierce Chemical Co., Rockford.

Other suitable methods for linking molecules to solid supports are well known to those of skill in this art [see, e.g., U.S. Patent No. 5,416,193].
30 These include linkers that are suitable for chemically linking molecules, such as proteins, to supports and include, but are not limited to, disulfide bonds, thioether bonds, hindered disulfide bonds, and covalent bonds between free

-136-

reactive groups, such as amine and thiol groups. These bonds can be produced using heterobifunctional reagents to produce reactive thiol groups on one or both of the moieties and then reacting the thiol groups on one moiety with reactive thiol groups or amine groups to which reactive maleimido groups or thiol groups can be attached on the other. Other linkers include, acid cleavable linkers, such as bismaleimideoxy propane, acid labile-transferrin conjugates and adipic acid dihydrazide, that would be cleaved in more acidic intracellular compartments; cross linkers that are cleaved upon exposure to UV or visible light and linkers, such as the various domains, such as C_H1, C_H2, and C_H3, from the constant region of human IgG, (see, Batra et al. (1993) Molecular Immunol. 30:379-386). Presently preferred linkages are direct linkages effected by adsorbing the molecule to the surface of the matrix. Other linkages are photocleavable linkages that can be activated by exposure to light [see, e.g., Goldmacher et al. (1992) Bioconj. Chem. 3:104-107, which linkers are herein incorporated by reference]. The photocleavable linker is selected such that the cleaving wavelength that does not damage linked moieties. Photocleavable linkers are linkers that are cleaved upon exposure to light [see, e.g., Hazum et al. (1981) in Pept., Proc. Eur. Pept. Symp., 16th, Brunfeldt, K (Ed), pp. 105-110, which describes the use of a nitrobenzyl group as a photocleavable protective group for cysteine; Yen et al. (1989) Makromol. Chem 190:69-82, which describes water soluble photocleavable copolymers, including hydroxypropylmethacrylamide copolymer, glycine copolymer, fluorescein copolymer and methylrhodamine copolymer; Goldmacher et al. (1992) Bioconj. Chem. 3:104-107, which describes a cross-linker and reagent that undergoes photolytic degradation upon exposure to near UV light (350 nm); and Senter et al. (1985) Photochem. Photobiol 42:231-237, which describes nitrobenzyloxycarbonyl chloride cross linking reagents that produce photocleavable linkages]. The selected linker will depend upon the particular application and, if needed, may be empirically selected.

-137-

These methods for linking molecules to supports may be adapted for use to link the targeting agents to the targeted agents.

M. Bioluminescence Resonance Energy Transfer (BRET) System

In nature, coelenterazine-using luciferases emit broadband blue-green
5 light (max. ~480 nm). Bioluminescence Resonance Energy Transfer (BRET) is a natural phenomenon first inferred from studies of the hydrozoan *Obelia* (Morin & Hastings (1971) J. Cell Physiol. 77:313-18), whereby the green bioluminescent emission observed *in vivo* was shown to be the result of the luciferase non-radiatively transferring energy to an accessory green fluorescent protein (GFP).
10 BRET was soon thereafter observed in the hydrozoan *Aequorea victoria* and the anthozoan *Renilla reniformis*. Although energy transfer *in vitro* between purified luciferase and GFP has been demonstrated in *Aequorea* (Morise et al. (1974) Biochemistry 13: 2656-62) and *Renilla* (Ward & Cormier (1976) J. Phys. Chem. 80:2289-91) systems, a key difference is that in solution efficient radiationless
15 energy transfer occurs only in *Renilla*, apparently due to the pre-association of one luciferase molecule with one GFP homodimer (Ward & Cormier (1978) Photochem. Photobiol. 27:389-96). The blue (486 nm) luminescent emission of *Renilla* luciferase can be completely converted to narrow band green emission (508 nm) upon addition of proper amounts of *Renilla* GFP (Ward & Cormier
20 (1976) J. Phys. Chem. 80: 2289-91). GFPs accept energy from excited states of luciferase-substrate complexes and re-emit the light as narrow-band green light (~510 nm). By virtue of the non-radiative energy transfer, the quantum yield of the luciferase is increased.

Luciferases and fluorescent proteins have many well-developed and
25 valuable uses as protein tags and transcriptional reporters; BRET has the potential to increase the sensitivity and scope of these applications. A GFP increases the sensitivity of the luciferase reporter by raising the quantum yield. A single luciferase fused to several spectrally distinct GFPs allows the simultaneous use of multiple luciferase reporters, activated by addition of a
30 single luciferin. By preparing two fusion proteins, each containing a GFP having a different emission wavelength fused to identical luciferases, two or more reporters can be used with a single substrate addition. Thus multiple events

-138-

may be monitored or multiple assays run using a single reagent addition. Such a reporter system is self-ratioing if the distribution of luciferin is uniform or reproducible.

The ability to conveniently monitor several simultaneous macromolecular events within a cell is a major improvement over current bioluminescent technology. BRET also enables completely new modes of reporting by exploiting changes in association or orientation of the luciferase and fluorescent protein. By making fusion proteins, the luciferase-GFP acceptor pair may be made to respond to changes in association or conformation of the fused moieties and hence serves as a sensor.

Energy transfer between two fluorescent proteins (FRET) as a physiological reporter has been reported [Miyawaki *et al.* (1997) *Nature* 388:882-7], in which two different GFPs were fused to the carboxyl and amino termini of calmodulin. Changes in calcium ion concentration caused a sufficient conformational change in calmodulin to alter the level of energy transfer between the GFP moieties. The observed change in donor emission was ~10% while the change in ratio was ~1.8.

The similar use of a luciferase-GFP pair in the presence of substrate luciferin as provided herein has important advantages. First, there is no background and no excitation of the acceptor from the primary exciting light. Second, because the quantum yield of the luciferase is greatly enhanced by nonradiative transfer to GFP, background from donor emission is less, and the signal from the acceptor relatively greater. Third, the wavelength shift from the peak emission of luciferase (~480 nm) to that of the GFP (typically 508-510 nm) is large, minimizing signal overlap. All three factors combine to increase the signal-to-noise ratio. The concentration of the GFP acceptor can be independently ascertained by using fluorescence.

For some applications, *in vitro* crosslinked or otherwise *in vitro* modified versions of the native proteins is contemplated. The genetically encoded fusion proteins have many great advantages: A) *In vivo* use - unlike chemistry-based luminescence or radioactivity-based assays, fusion proteins can be genetically incorporated into living cells or whole organisms. This greatly increases the

-139-

range of possible applications; B) Flexible and precise modification - many different response modifying elements can be reproducibly and quantitatively incorporated into a given luciferase-GFP pair; C) Simple purification - only one reagent would need to be purified, and its purification could be monitored via the fluorescent protein moiety. Ligand-binding motifs can be incorporated to facilitate affinity purification methods.

1. Design of sensors based on BRET

Resonance energy transfer between two chromophores is a quantum mechanical process that is exquisitely sensitive to the distance between the donor and acceptor chromophores and their relative orientation in space (Wu & Brand (1994) Anal. Biochem. 218 1-13). Efficiency of energy transfer is inversely proportional to the 6th power of chromophore separation. In practice, the useful distance range is about 10 to 100 Å, which has made resonance energy transfer a very useful technique for studying the interactions of biological macromolecules. A variety of fluorescence-based FRET biosensors have been constructed, initially employing chemical fluors conjugated to proteins or membrane components, and more recently, using pairs of spectrally distinct GFP mutants (Giuliano & Taylor (1998) Trends Biotech. 16: 99-146; Tsien (1998) Annu. Rev. Biochem. 67:509-44).

Although these genetically encoded GFP bioluminescence -based biosensors have advantages over less convenient and less precise chemical conjugate-based biosensors, all share a limitation in their design: it is generally difficult to construct a biosensor in which energy transfer is quantitative when the chromophores are in closest apposition. It is almost impossible to arbitrarily manipulate the complex stereochemistry of proteins so that conjugated or intrinsic chromophores are *stably positioned with minimal separation and optimal orientation*. The efficiency of such biosensors are also often limited by stoichiometric imbalances between resonance energy donor and acceptor; the donor and acceptor macromolecules must be quantitatively complexed to avoid background signal emanating from uncomplexed chromophores. These limitations in general design become important when biosensors must be robust, convenient and cheap. Developing technologies such as high throughput

-140-

screening for candidate drugs (using high throughput screening (HTS) protocols), biochips and environmental monitoring systems would benefit greatly from modular biosensors where the signal of a rare target "hit" (e.g., complex formation between two polypeptides) is unambiguously (statistically) distinguishable from the huge excess of "non-hits"). Current genetically encoded FRET and bioluminescence-based biosensors display hit signals that very often are less than two-fold greater than non-hit signals, and are at best a few-fold greater (Xu et al. (1999) Proc. Natl. Acad. Sci USA 96: 151-156; Miyawaki et al. (1997) Nature 388:882-7).

10 To solve these problems, the anthozoan GFPs, such as the *Renilla* GFPs, provided herein can be used in combination with their cognate luciferases. Anthozoan luciferases-GFP complexes provide a "scaffold" upon which protein domains that confer the biological properties specific to a given biosensor can be linked. Although one can construct many useful two component biosensors based on this scaffold, in a biosensor contemplated herein, independent protein domains that potentially complex with one another are respectively fused to the luciferase and the GFP.

20 In isolation, an anthozoan luciferase emits blue light from the coelenterazine-derived chromophore (A), and an anthozoan GFP that is excited with blue-green light emits green light from its integral peptide based fluorophore (B). When the luciferase and GFP associate as a complex *in vivo* or *in vitro*, the luciferase non-radiatively transfers its reaction energy to the GFP fluorophore, which then emits the green (C). Thus any molecular interaction that disrupts the luciferase-GFP complex can be quantitatively monitored by observing the ratio of blue to green light (D).

25 There are many possible variations on this theme. For example, in a three component system either the luciferase or GFP can be fused to a ligand-binding domain from a protein of interest or other target peptide or other moiety of interest. If the design of the fusion protein is correct, binding of a small molecule or protein ligand then prevents the luciferase-GFP association, and one has a BRET-based biosensor. More complex protein fusions can be designed to

-141-

create two component and even single component BRET biosensors for a multitude of uses.

FIGURE 11 illustrates the underlying principle of Bioluminescent Resonance Energy Transfer (BRET) and its use as sensor: A) in isolation, a
5 luciferase, preferably an anthozoan luciferase, emits blue light from the coelenterazine-derived chromophore; B) in isolation, a GFP, preferably an anthozoan GFP that binds to the luciferase, that is excited with blue-green light emits green light from its integral peptide based flurophore; C) when the
— luciferase and GFP associate as a complex *in vivo* or *in vitro*, the luciferase non-
10 radiatively transfers its reaction energy to the GFP flurophore, which then emits the green light; D) any molecular interaction that disrupts the luciferase-GFP complex can be quantitatively monitored by observing the spectral shift from green to blue light.

15 The nucleic acids, and the constructs and plasmids herein, permit preparation of a variety of configurations of fusion proteins that include an anthozoan GFP, such as *Renilla*, with its cognate anthozoan luciferase. The nucleic acid encoding the GFP can be fused adjacent to the nucleic acid encoding the luciferase or separated therefrom by insertion of nucleic acid
20 encoding, for example, a ligand-binding domain of a protein of interest. The GFP and luciferase will be bound. Upon interaction of the ligand-binding domain with the a test compound or other moiety, the interaction of the GFP and luciferase will be altered thereby changing the emission signal of the complex. If necessary the GFP and luciferase can be modified to fine tune the interaction
25 to make it more sensitive to conformational changes or to temperature or other parameters.

2. Advantages of BRET sensors

There are many advantages to the BRET senors provided herein. For example, BRET sensors are self-ratioing. The reporter and target are integrated
30 into single polypeptide. This ensures 1:1:1 stoichiometry among luciferase, GFP and target (or a 1:N:1 stoichiometry if more than one, typically a homodimer, GFP can be bound to a luciferase). GFP fluorescence allows

-142-

absolute quantitation of sensor. The null state gives signal that verifies sensor functionality. Quantifiable null state facilitates disruption-of-BRET sensors (DBRET). BRET sensors have better signal-to-noise ratio than GFP FRET sensors because there is no cellular autofluorescence, no excitation of the acceptor from the primary exciting light, the quantum yield of luciferase greatly enhanced by non-radiative energy transfer to GFP, and there is minimal signal overlap between emission of the luciferase and emission of the GFP. Also, anthozoan GFPs have 6-fold higher extinction coefficients than Aequorea GFP.

The BRET sensors can for used for hit identification and downstream evaluation in *in vitro* screening assays in *in vitro* or *in vivo* or *in situ*, including in cultured cells and tissues and animals. The BRET sensors can be created by thermal endpoint-selection, which is suited to DBRET (Disruption-of-BRET) and reduces need for knowledge of target 3D structure and functional dynamics. Existing screening robotics to optimize biosensors. BRET sensors benefit from vast genetic diversity anthozoans have evolved efficient luciferase-GFP energy transfer systems and the components can be mixed and matched. Highly efficient heterologous luciferases may be substituted for less active luciferases. For example, a copepod luciferase active site can be fused to an anthozoan luciferase GFP-binding domain. There are many diverse coelenterazine-using luciferases.

BRET sensors are modular so that an optimized sensor scaffold may be used with different targets. Also the BRET acceptor may be varied to give shifted emissions, facilitating multiple simultaneous readouts. The anthozoan GFPs can be mutated, GFPs or other proteins can be modified with different chemical fluors, high throughput screening (HTS) fluor-modified FRET acceptors can be adapted, the BRET donor (luciferase) may be varied, such as by using an Aequorin (Ca⁺⁺ activated) photoprotein, or a firefly luciferase (requires ATP and a firefly luciferin) to give conditional activation. The sensor scaffold can be incorporated into a variety of immobilization motifs, including free format plates, which can reduce reagent volumes, reusable microtiter plates, miniature columns

-143-

and biochips. Finally, BRET sensors are inexpensive and reproducible reagents because they can be produced by standardized protein production and can incorporate purification tags. Genetically encoded reporters more reproducible than chemically modified reporters. Linear translation of BRET modules ensures
5 sensor integrity.

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLE 1

10 I. TOXICOLOGY

1. Solubility of Coelenterazine

Coelenterazine is not terribly soluble in non-irritant vehicles.

Coelenterazine is soluble to at least to a concentration of 200 micrograms/mL in a solution of 2% (w/v) PEG 400 containing about 0.8% (w/v) NaCl. Although
15 this solution is slightly hypertonic, it is not an irritant for vehicle purposes.

2. Toxicology of Coelenterazine

A. Topical Administration

To examine the toxicology of the above-described coelenterazine solution, the solution was administered in the eyes of anesthetized rabbits
20 following standard procedures and conjunctival irritation was measured. Animals were sedated with diazepam (about 2 mg/kg) and 100 μ L of the coelenterazine in the PEG solution was instilled in one eye and to the other eye only the PEG vehicle was instilled. Animals were observed for a 30-min period and then the animals were carefully examined for any conjunctival irritation as
25 well as any corneal ulceration. The examination was performed using a slit-lamp to visualize the eye well. Only minimal conjunctival irritation from the vehicle was observed in either eye (n=3). Thus, the direct administration to the eye of about 20 μ g of coelenterazine in this solution produced no irritation, ulceration, or other signs of toxicity in this topical assay.

B. Intravenous Administration

30 In a second experiment, mice are administered coelenterazine (n=6) at a concentration 1 mg/kg, i.p. or vehicle (n=6) for a seven-day period. Mice are

-144-

examined over the course of the study for any gross signs of toxicity as evidenced in their behavior.

At the end of the one-week period, blood is collected by cardiac puncture immediately prior to sacrifice. Animals are sacrificed and ten different
5 tissue samples are removed post mortem from each animal. Isolated tissues are fixed, stained, blocked and sectioned. The pathology of the tissue samples are analyzed and the toxicology data are compiled. Daily administration of coelenterazine for three days resulted in no gross behavior changes in the test animals.

10 **C. Stability of Coelenterazine**

The stability of coelenterazine may be determined by analyzing biological samples for the presence of coelenterazine and metabolic products derived therefrom. In this experiment, blood will be collected and serum prepared, and this serum can be assayed for coelenterazine and its metabolites. Little
15 interference was observed from the serum (mouse) at the emission wavelength requisite for coelenterazine.

Alternatively, a lobe of liver may be resected from each animal and separately pooled, fixed, homogenized in cold acid acetone, and assayed for coelenterazine and its metabolites by standard biochemical analyses.

20 **D. Coelenterazine Assays**

The concentration of coelenterazine may be determined using its inherent fluorescence properties. For example, coelenterazine may be measured in an alcohol solution by measuring the fluorescence at a specified wavelength. To date, the detectable limit is less than 10 ng/mL. Given the dosages
25 contemplated herein, this level of sensitivity should be sufficient for accurate measurement.

The concentration of coelenterazine may also be determined by use of HPLC in combination with fluorescence detection. In addition to an HPLC-based detection system, coelenterazine and its metabolites may be identified by Gas
30 Chromatography (GC) or by Mass Spectrophotometry analysis. Final confirmation of the identity of coelenterazine and its metabolites may be performed by nuclear magnetic resonance (NMR).

-145-

Method of Preparing Photoprotein Conjugates

A method for the preparation of photoprotein conjugates that retain bioluminescent activity has been described (see U.S. Patent No. 5,486,455). In general, additional sulfhydryl groups are introduced into the photoprotein by
5 treatment of the photoprotein with Trauts Reagent (2-iminothiolane) to generate a sulfhydryl-activated photoprotein. The sulfhydryl-activated photoprotein is conjugated to a sulfhydryl-reactive binding reagent (e.g., a macromolecule that has been chemically modified with a heterobifunctional linker that is capable of sulfhydryl crosslinking, such as maleimido- or sulfo-SMCC, sulfosuccinimidyl-4-
10 (N-maleimidomethyl) cyclohexane-1-carboxylate). The conjugated photoprotein may be used in crude form or may be further purified by methods known to those of skill in the art, such as ion exchange or affinity chromatography.

EXAMPLE 2

Rodent Model

15 A monoclonal antibody directed against a human tumor antigen (e.g., Lewis antigen or carcinoembryonic antigen [CEA]) or a humanized derivative thereof is conjugated to a photoprotein, preferably aequorin, or to the vargula luciferase, via the sulfhydryl-binding method (see U.S. Patent No. 5,486,455) and the conjugate is purified. Approximately, 10-100 micrograms of the
20 antibody-photoprotein conjugate is injected i.v. in the tail vein of a transgenic mouse which expresses a human tumor antigen. The injection should be tolerated well by the animal.

After sufficient time is allowed for antibody binding (2-48 hours), approximately 1 μ g of the coelenterazine or 10 μ L of crude lysate containing the
25 remaining bioluminescence generating components is injected i.p. directly in the region of the proposed neoplasm. Alternatively, 10 μ L of the lysate or 1 μ g of coelenterazine is i.p. injected and time allowed for the coelenterazine to circulate to the target region (25 minutes to 2 days).

The mouse is then anesthetized and the region containing the neoplasm
30 is exposed in a dark room. Regions that emit light as determined by a photometer or by the human eye are targeted for surgical removal. Alternatively, the region of interest may be visualized by the insertion of a

-146-

laparoscope near the site of the neoplasm and subsequent placement of the imaging camera in a position to observe light.

EXAMPLE 3

ISOLATION AND IDENTIFICATION OF DNA ENCODING *Renilla mulleri* GFP

5 1. Preparation of a *R. mulleri* cDNA expression library

A *R. mulleri* cDNA expression library was prepared using the commercially available Lambda-UniZap XR Vector kit (Stratagene) according to the direction provided. Briefly, EcoRI and XhoI adaptors were ligated to 5'-end of the cDNA fragments and the ligated cDNA fragments were purified from the remaining unligated adaptors. The purified cDNAs were ligated into EcoRI- and XhoI-digested λ Uni-ZAP XR vector, transformed into competent *E. coli* XL-1 Blue cells and the resulting DNA was packaged into viral particles using λ phage helper extracts (Gigapak Plus Kit, Stratagene). The packaged lambda library was titered in *E. coli* XL-1 Blue cells and the sequence complexity of the *Renilla mulleri* cDNA expression library was calculated to be about 1.73×10^6 independent plaques.

A plasmid library was derived from the lambda cDNA expression library by excision of the initiator-terminator cassette harboring the cloned *Renilla* cDNA. Approximately 2×10^8 independent plaque isolates were pooled and used to infect *E. coli* SOLR cells (Stratagene), which were then co-infected with a filamentous helper phage VCSM13, R408 or ExAssist helper phage (Stratagene). The cDNA-containing plasmids were recovered by plating the infected cells on solid medium supplemented with 200 μ g/ml ampicillin for the selection of cells containing excised pBK plasmid.

25 In *E. coli* XL-1 Blue cells, the expression of the *Renilla mulleri* GFP in the pBK plasmid is under the control of the lacZ promoter, whose transcription is easily induced by the addition of isopropylthio- β -D-galactopyranoside (IPTG) to the culture medium or may be applied directly to the colonies in spray form or other aerosols.

30 2. cDNA library screening

To identify clones expressing a *Renilla* GFP, a functional screening method employing blue light, e.g., 490 nm, was used to identify fluorescent

-147-

GFP transformants expressing a *Renilla* GFP. The *Renilla* cDNA expression plasmid library was screened by transforming competent *E. coli* XL-1 Blue cells and plating a portion of the transformation mixture on L-broth plates supplemented with 200 μ g/ml ampicillin containing carbon black, which was added to completely absorb background fluorescence (e.g., from the agar). Plates were illuminated with narrow bandwidth light centered at 490 nm and observed through a 510 nm narrow bandpass filter using methods generally known in the art (e.g., see Ward *et al.* (1978) J. Biol. Chem. 254: 781-788).

Approximately $3-4 \times 10^6$ individual colonies were screened and a three light emitting colony were identified. To confirm that the above-described strain harbored a plasmid that encoded a GFP, the spectral properties of the plasmid-encoded protein were assessed using cell lysates and partially purified cell extracts (e.g., see EXAMPLE 4). The fluorescence excitation spectrum for partially purified recombinant *Renilla mulleri* GFP was similar to those reported for other *Renilla* species (maximum near 498 nm); however, the recombinant *R. mulleri* GFP emission spectrum has a wavelength maximum near 506 nm, which is a slightly shorter wavelength maximum than the in vitro and in vivo emission spectrum obtained for naturally-occurring *Renilla* GFP (e.g., 509 nm; see Wampler *et al.* (1973) Biochem. Biophys. Acta 314:104-109).

3. Determination and characterization of the nucleotide sequence of DNA encoding *Renilla mulleri* GFP

Plasmid DNA was purified from cultures of the fluorescent transformant and the nucleotide sequence of the *Renilla* cDNA plasmid insert was determined using methods well known to those of skill in the art (e.g., see Sambrook *et al.* (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Sanger *et al.* () Proc. Natl. Acad. Sci. U.S.A.).

The nucleotide sequence of a cDNA encoding a full-length *Renilla mulleri* GFP is set forth in SEQ ID No. 15. The cDNA fragment encoding the *Renilla mulleri* GFP is 1,079 nt in length, including 258 nt of 5' noncoding sequence, a 714 nt open reading frame, encoding a 238 amino acid polypeptide, and 107 nt of 3' non-coding sequence.

-148-

- The nucleotide sequence of the cDNA encoding the *Renilla* GFP was compared to the nucleotide sequence of the *A. victoria* GFP, the only other GFP whose complete nucleotide sequence is known (e.g., see SEQ ID No. 1). The nucleic acids isolated from the two organisms encode proteins of identical length, however, the nucleotide sequence that encodes the amino-terminal 136 amino acid residues of the *Renilla mulleri* GFP is only 48.8% identical compared to *A. victoria*. Furthermore, the nucleotide sequence encoding the carboxy-terminal 102 amino acid residues of *Renilla mulleri* GFP is even more highly divergent, being only 31.4% identical.
- 10 A comparison of the deduced amino acid sequences of the *Renilla mulleri* GFP and the *A. victoria* GFP revealed the protein sequences are also highly divergent. Only 56 of 238 amino acid residues between the deduced amino acid sequences are identical (i.e., 23.5% direct amino acid identity). Moreover, the deduced sequence of the putative hexapeptide chromophore in *R. mulleri*
- 15 (FQYGNR) is quite different from that of *A. victoria* (FSYGVQ) having only 3 out of 6 identical amino acid residues. The *Renilla mulleri* chromophore is also located in a slight different position in the polypeptide chain compared to *A. victoria* GFP. The *R. mulleri* chromophore is encoded by amino acid residues 68-73 whereas the *A. victoria* chromophore is encoded by amino acid residues
- 20 64-69. The slightly different position and altered chromophore sequence likely contribute to the differing spectral properties exhibited by the two proteins.

EXAMPLE 4

25 IDENTIFICATION AND ISOLATION OF DNA ENCODING A *Renilla mulleri* LUCIFERASE

- The *R. mulleri* cDNA plasmid library described in EXAMPLE 3 was transformed in *E. coli* XL-1 Blue cells and single colonies were obtained by plating a portion of the transformation mixture on L-broth plates supplemented
- 30 with 200 μ g/ml ampicillin and also supplemented with carbon black to absorb background fluorescence. The plates were incubated overnight at 37°C. Ampicillin resistant transformants were sprayed with a 1 mM IPTG solution to induce luciferase expression. After allowing time for the cells to express the

-149-

luciferase, the surface of the plates were sprayed with a solution containing 20 mM coelenterazine and colonies emitting blue light were visualized using a blue bandwidth filter. Plasmid DNA was isolated from cultures of bioluminescent transformants and the nucleotide sequence of a cDNA insert of a positive clone
5 was determined. The nucleotide sequence of DNA encoding a full-length *Renilla mulleri* luciferase and the deduced amino acid sequence are set forth in SEQ ID No. 17. The cDNA fragment encoding the *Renilla mulleri* luciferase is 1,217 nt in length, including 30 nt of 5'-non-coding region, a 933 nt open reading frame encoding a 311 amino acid polypeptide and 254 nt of 3'-non-coding sequence.

10

EXAMPLE 5

RECOMBINANT PRODUCTION *Renilla* LUCIFERASE1. Recombinant production of *Renilla reniformis* luciferase

The phagemid pTZ18R (Pharmacia) is a multi-purpose DNA vector designed for in vitro transcriptions and useful for expression of recombinant
15 proteins in bacterial hosts. The vector contains the bla gene, which allows for the selection of transformants by resistance to ampicillin, and a polylinker site adjacent to the lacZ' gene. The heterologous gene of interest is inserted in the polylinker and transcribed from the lac promoter by induction, for example, with isopropyl- β -D-thiogalactopyranoside (IPTG).

20 The DNA encoding the *Renilla reniformis* luciferase has been cloned (e.g., see U.S. Patent Nos. 5,292,658 and 5,418,155). The plasmid pTZRLuc-1 encodes the *Renilla* luciferase on a 2.2 Kbp EcoRI to SstI DNA fragment inserted in EcoRI and SstI sites of pTZ18R (plasmid construction is described U.S. Patent Nos. 5,292,658 and 5,418,155; see also Lorenz et al. (1991) Isolation and
25 Expression of a cDNA encoding *Renilla reniformis* Luciferase, Proc. Natl. Acad. Sci. U.S.A. 88, 4438-4442). The initiation of transcription of the *Renilla* luciferase cDNA is under the control of the lacZ' promoter. E. coli strains harboring plasmid pTZRLuc-1 express *Renilla* luciferase that is functional in bioluminescence assays and retains the properties of the native enzyme (see
30 e.g., U.S. Patent Nos. 5,292,658 and 5,418,155).

A derivative of pTZRLuc-1, pTZRLuc-3.6, produces approximately 7-fold higher levels of recombinant *Renilla* luciferase than pTZRLuc-1 when

-150-

transformed into the same *E. coli* host. Competent *E. coli* strain XL-1 was transformed using purified pTZRLuc-3.6 according to the instructions provided by the manufacturer (XL-1 Supercompetent cells and protocol; Stratagene, Inc., La Jolla, CA). Transfectants were selected by plating on Luria Broth (LB) plates
5 supplemented with 100 µg/ml ampicillin.

Single ampicillin resistant colonies were grown in LB medium supplemented with 100 µg/ml ampicillin at ambient temperature using continuous shaking until cell growth reached mid-log phase (i.e., cell culture reaches an O.D._{600nm} = 0.6-0.8 units). Transcription from the *lac* promoter was
10 induced by addition of 1 mM IPTG and cell culture was shaken at ambient temperature for an additional 8 hours.

Cells were harvested by centrifugation at 10,000 x g and frozen at -20° C. The cell pellet was thawed and resuspended at a 1:5 ratio (w/w) in a solution of 10 mM EDTA, pH 8.0, containing 4 mg/ml lysozyme (Sigma
15 Chemical Corp.). The cells were placed in a 25° C water bath for 30 minutes and then transferred to ice for 1 hour. The cells were lysed by sonication at 0° C using a 1 minute pulse from an Ultrasonics, Inc. cell disrupter.

The lysed cellular debris was removed by centrifugation at 30,000 x g for 3 hours and the supernatant was decanted and retained. The pellet was
20 resuspended at a 1:5 ratio in the above-described solutions, and the subsequent incubations, lysis and centrifugation steps were repeated. The two supernatants were combined and stored at -70° C. The resulting "clarified lysate" was employed as a source of recombinant luciferase. Alternatively, the lysate may be subjected to additional purification steps (e.g.,
25 ion exchange chromatography or immunoaffinity chromatography) to further enrich the lysate or provide a homogeneous source of the purified enzyme (see e.g., U.S. Patent Nos. 5,292,658 and 5,418,155).

Alternatively, recombinant *Renilla mulleri* luciferase may be expressed by substituting the DNA encoding the *R. reniformis* luciferase for the DNA
30 encoding a *R. mulleri* luciferase, such as DNA encoding the sequence of amino acids set forth in SEQ ID No. 18.

EXAMPLE 6

-151-

DETECTION OF CANCER CELLS

The luciferase-based bioluminescent detection method has broad application in the visualization and precise localization of cancer cells. In such applications, the *Renilla* GFP, *Renilla mulleri* luciferase or luciferin molecule may be conjugated to a targeting agent, such as an anti-tumor antigen antibody, which specifically recognizes certain cancer cells that express the antigen. Alternatively, the luciferase is coupled to a microcarrier and the targeting agent is conjugated to the luciferase and/or the microcarrier. The conjugate is introduced into a subject, for example, through intravenous, intraperitoneal or subcutaneous injection or through topical application or direct application during surgery using a laparoscope or trocar. Through formation of an antibody-antigen complex, the luciferase or luciferin is linked to the target cancer cells and available for interaction with luciferin substrate (if the conjugate contains luciferase) or luciferase enzyme (if the conjugate contains luciferin). Thus, the substrate or enzyme is then introduced into the subject, e.g., through injection or application, and allowed to react with the partner molecule contained in the antibody conjugate to yield the readily detectable light emission only the precise areas where the conjugate is stably present as an antibody-antigen complex.

The sensitivity and biocompatibility of this bioluminescence detection system make it possible to discover cancer in its early stages, e.g., small numbers of cancer cells, in contrast to other less sensitive methods which are able to detect cancer cells only after the neoplasm has developed to a more advanced and potentially life-threatening stage. In addition, diagnostic methods disclosed herein may be utilized in the absence of invasive surgical procedures. For example, surgical viewing devices, computer tomograms or miniature surgical viewing instruments, see above, that has been modified to detect low intensity levels of visible red and near infrared light emitted through the tissues of the patient may also be used to assist the surgeon, such as those described in co-pending, co-owned U.S. application Serial No. 08/990,103.

The bioluminescence detection system is particularly applicable in surgical procedures to remove cancerous lesions. The targeting of a *Renilla* GFP, *Renilla mulleri* luciferase and/or luciferin to, e.g., a tumor, results in the

-152-

precise delineation of the boundaries of the lesion and thereby facilitates a more exact excision and complete eradication of the cancer without removal of surrounding healthy tissue. This is of critical importance in the excision of cancerous lesions in complex, vital tissues, such as nervous tissue. The sensitivity of the bioluminescence generating system also makes it well-suited for post-surgery evaluations and identification of metastases in which the ability to detect small numbers of any remaining cancer cells enables a more accurate assessment of the effectiveness of the procedure in eradicating the cancer.

The bioluminescence generating system finds further use in monitoring the progression and spread of cancer. Such information is invaluable in assessing the effectiveness of therapies, such as chemotherapy and radiation therapy, as well as the efficacy of drug-based therapies in treating cancer patients.

Detection of Cervical Cancer

A luciferase-based bioluminescence detection system can be used in the detection of cervical cancer. For example, luciferin or luciferase may be conjugated, directly or through a linker or microcarrier, to antibodies specific for cervical cancer cell antigens (e.g., see Table 3). The conjugate is then directly applied in an appropriate formulation to the cervical tissue which is then rinsed to eliminate any unbound conjugate. The remaining components of the bioluminescent reaction, i.e., luciferin if the conjugate contains luciferase or luciferase if the conjugate contains luciferin, is then applied to the cervical tissue, along with any necessary activators, and allowed to interact with any bound conjugate. Light emission is then monitored. The light emitted may be of any visible wavelength detectable by a human eye. If cancer cells presenting the recognized antigen are present in the tissue, those cells will glow and thereby be visualized. The bioluminescence serves to provide a more precise localization of the cancer which guides a surgeon in removal of the cancerous lesion.

-153-

Detection of Carcinoembryonic Antigen (CEA)

A luciferase-based bioluminescence detection system can also be used in the detection of neoplastic cells presenting CEA, such as, e.g., cancerous cells present in colorectal cancers (e.g., see Table 3). In this application, the
5 luciferase or luciferin is conjugated, directly or indirectly, to an antibody specific for CEA, and detection is accomplished as described above for the detection of cervical cancer. The migration of CEA-bearing cancer cells, for instance into the wall of the colon and further into the lymphatics, may also be monitored with this detection system. The modified laparoscope that detects low intensity
10 visible light may be further employed to enhance the detection and visualization of the CEA-bearing cancer cells.

Detection of Urinary Bladder Cancer

For detection of urinary bladder cancers, the luciferase or luciferin is conjugated to a targeting agent, e.g., an antibody that recognize antigens
15 presented on bladder cancer cells, that serve to link the conjugate to the cancerous lesions. The conjugate is introduced into the bladder, for example through a catheter, and the lesions are visualized and delineated upon subsequent introduction of the remaining components of the bioluminescent reaction into the bladder. This embodiment is particularly useful for urinary
20 cancers of the bladder, which are currently removed during surgery by transurethral burning of the tumor located in the bladder wall using an electro-cautery. This technique would minimize cauterization of healthy bladder tissue, identify potential areas of metastasis and ensure complete surgical removal of the target.

25 In another embodiment, the location and margins of neoplastic bladder tissue may be defined with greater particularity by detecting the presence of the tumor with targeting agent coupled to the luciferase-bound microparticle. After administration of the target agent conjugate, the bioluminescent reaction is initiated (i.e., by addition of a luciferin and/or any activators). A secondary,
30 GFP-bound microparticle is covalently linked to a targeting agent which is directed against nearby surrounding tissue or which preferentially targets identical, non-tumorigenic tissue. The GFP conjugate is administered to the

-154-

patient. Thus, for example, the neoplastic tissue would glow emitting a blue light, e.g., using aequorin or *Renilla* luciferase-targeting agent conjugate whereas the GFP-bound surrounding tissue would absorb the blue light and emit green light thereby providing additional contrast to clearly define the margins of the tissue to be surgically removed.

Detection of the Spread of Migratory Cancer Cells

The infiltration of the lymphatic system by migratory cancer cells, such as from cutaneous melanomas, deep breast tumors and hepatic metastases originating in colon cancer, may be readily detected using the bioluminescence detection system. The luciferase or luciferin conjugated to a targeting agent, such as an antibody that recognizes a cancer cell antigen, complexes specifically with the cells, no matter where they are in the migratory process. The remaining bioluminescence generating components are then allowed to circulate throughout the body to interact only with the cells to which conjugate is bound. In instances in which the cancer cells have invaded the epithelial tissues at or near the surface of the skin, the conjugate and/or partner molecule may be topically applied and the resulting light emission readily detected by the human eye without invasive procedures. Additionally, a photomultiplier or surgical viewing devices may also be used to amplify the light output through the skin. In this manner, it may be possible to trace lymphatic migration of tumor cells before surgery is attempted.

Detection of Breast Cancer

The benefits of early detection of breast cancer, e.g., increased survivability rate and greater options for treatment, are numerous and well documented. The bioluminescence detection system provides a sensitive method to facilitate early diagnosis of breast cancers. For example, in such applications, the luciferase or luciferin may be conjugated to anti-estrogen or anti-progesterone receptor antibodies which target molecules that are greatly increased in number in breast cancer tissue as opposed to normal breast tissue. Thus, in this essentially quantitative assay system, the diagnosis depends on the level of luminescence detected, for example, in biopsied breast tissue. The

-155-

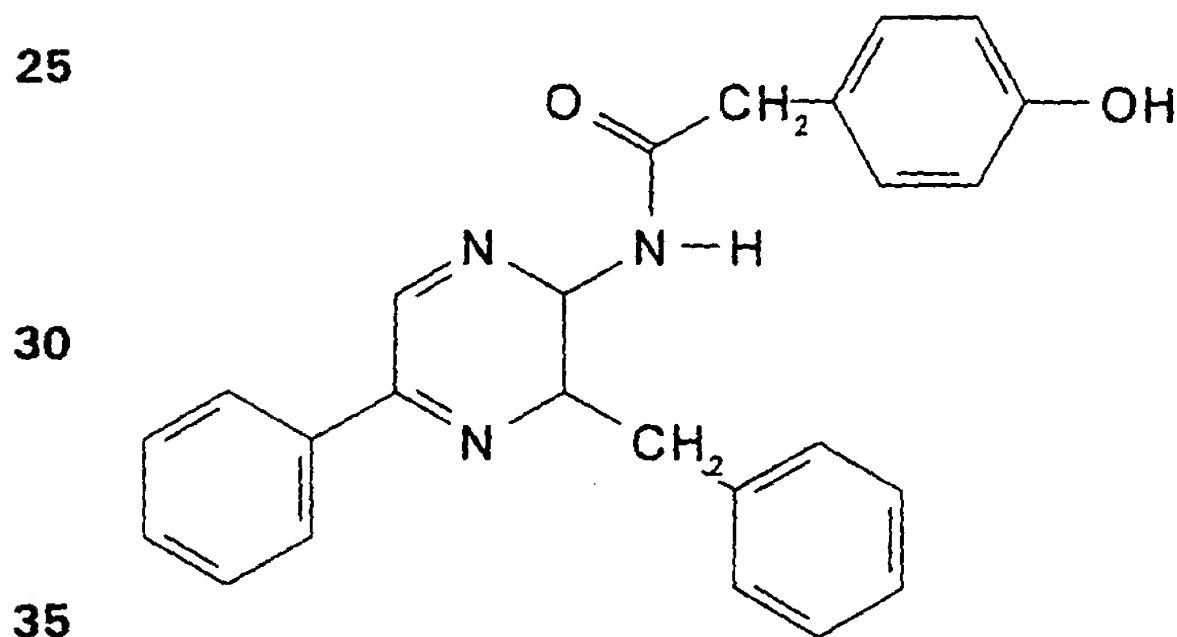
level of luminescence may be quantified using a photometer, photomultipliers or other suitable means.

Alternatively, the targeting agent may be coupled directly or indirectly to the luciferase isolated from *Aristostomias*, *Malacosteus* or *Pachystomias*, which
5 emit red light [e.g., see Widder *et al.* (1984) *Science* 225:512-514]. Particularly preferred are bioluminescence components isolated from the species *Aristostomias scintillans* and *Malacosteus niger*. In this application, the luciferase-containing targeting agent is administered to the patient followed by
10 the remaining components of the bioluminescence generating system (e.g., a luciferin and/or activators). Light emissions in this wavelength are detected directly through the tissue without an invasive surgical procedure using a photomultiplier, computer tomograph or using surgical vision device that is highly sensitive to red light. Alternatively, a surgical viewing instrument may be
15 used in which the optical detector means contains a CCD imager or an image intensifier that is particularly sensitive to red light emissions.

EXAMPLE 7

AMPLIFICATION OF FLUORESCENT EMISSIONS USING *RENILLA* GFP

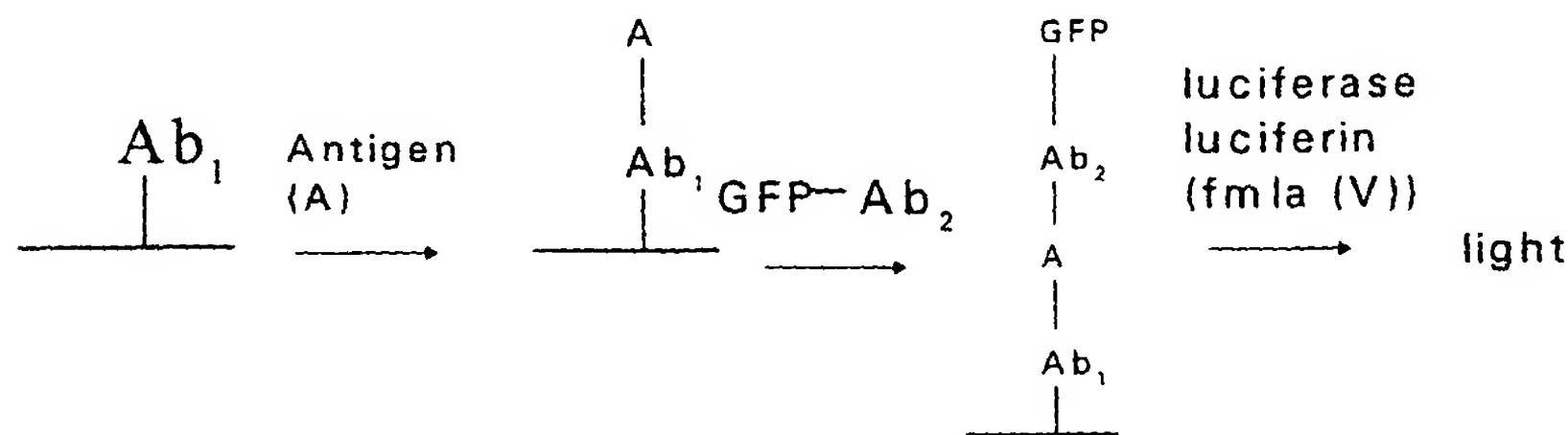
In the presence of *Renilla* GFP, the bioluminescence quantum yield of the *Renilla* luciferase-coelenterazine reaction is increased from 6.9% to 13%, an
20 amplification of nearly two-fold. Derivatives of coelenterazine in which one or more group of the coelenterazine structure have been replaced are known (e.g., see Hart *et al.* (1979) *Biochemistry* 18:2004). Of particular interest herein is coelenterazine of formula (V), discussed above:



-156-

As noted, reaction of this compound in the presence of *Renilla* luciferase produces ultraviolet light, λ maximum 390 nm, and the bioluminescence quantum yield is relatively low (about 0.012%). Upon addition of GFP, however, the *Renilla* luciferase/GFP complex emits green light and the bioluminescence quantum yield is increased to 2.3%. Therefore, the addition of GFP results in an approximate 200-fold increase in the amount of light emitted by *Renilla* luciferase. Furthermore, using a bandpass filter with a exclusion limit of less than 470 nm, only those wavelengths of light greater than 470 nm can be observed. Under these conditions, the visualization of light emissions is directly dependent on the presence of a GFP in order to shift the blue photons of light to those greater than 470 nm (e.g., 510 nm green light).

The use of *Renilla* GFP in combination with a luciferase that emits blue light and this coelenterazine derivative and the bandpass filter allows for the development of immunoassays in which detectable light production is dependent upon the presence of a GFP. A number of configurations for such immunoassays are possible and an exemplary immunoassay for use in which the reaction is performed on a solid support, such as a microtiter array format is as follows:



20

When used herein, a test sample suspected of containing the target antigen(s) is added to a microtiter plate containing a plurality of antibodies specific for a targeted antigen(s) that are individually attached to the wells. After forming an antibody-antigen complex, an antibody conjugate containing a secondary antibody specific for the antigen, or $F(Ab)_2$ fragment thereof, that is

25

-157-

linked to a *Renilla* GFP, such as those described herein is added. Specifically bound secondary antibody is detected by the addition of a luciferase, preferably a *Renilla reniformis* or *Renilla mulleri* luciferase, and the compound of formula (V) and light production is observed using a 470 nm bandpass filter. The light intensity should be a measure of the amount of GFP-Ab₂ present, which in turn is a measure of the amount of antigen bound to Ab₁.

Using this system, the presence of the antigen in the sample is confirmed by detecting light for the individual wells to which the antibody is specific. By knowing the specificity of the antibody, the specific antigen present in the sample can be identified. Thus, it should be possible to perform immunoassays that do not require an intermediate washing step prior to the addition of luciferase/luciferin.

EXAMPLE 8

IDENTIFICATION AND ISOLATION OF DNA ENCODING A *Gaussia mulleri* LUCIFERASE

1. Preparation of a *Gaussia* DNA expression library

A *Gaussia* cDNA expression library was prepared using the commercially available Lamda-UniZap XR Vector kit (Stratagene) according to the directions provided. Briefly, EcoRI and XhoI adaptors were ligated to 5'-end of the cDNA fragments and the ligated cDNA fragments were purified from the remaining unligated adaptors. The purified cDNAs were ligated into EcoRI- and XhoI-digested λ Uni-ZAP XR vector, transformed into competent *E. coli* XL-1 Blue cells and the resulting DNA was packaged into viral particles using λ phage helper extracts (Gigapak Plus Kit, Stratagene). The packaged lambda library was titrated in *E. coli* XL-1 Blue cells and the sequence complexity of the cDNA expression library was calculated.

A plasmid library was derived from the lambda cDNA expression library by excision of the initiator-terminator cassette harboring the cloned *Gaussia* luciferase-encoding DNA. Approximately 2×10^8 independent plaque isolates were pooled and used to infect *E. coli* SOLR cells (Stratagene), which were then co-infected with a filamentous helper phage VCSM13, R408 or ExAssist helper phage (Stratagene). The cDNA-containing plasmids were recovered by plating

-158-

the infected cells on solid medium supplemented with 200 μ g/ml ampicillin for the selection of cells containing excised pBK plasmid.

In *E. coli* XL-1 Blue cells, the expression of the DNA encoding the luciferase in the pBK plasmid is under the control of the lacZ promoter, whose
5 transcription is easily induced by the addition of isopropylthio- β -D-galactopyranoside (IPTG) to the culture medium or may be applied directly to the colonies in spray form or other aerosols.

2. cDNA library screening

To identify clones expressing a *Gaussia* luciferase, a functional screening
10 method was used. The cDNA plasmid library transformed into *E. coli* XL-1 Blue cells and single colonies were obtained by plating a portion of the transformation mixture on L-broth plates supplemented with 200 μ g/ml ampicillin and also supplemented with carbon black to absorb background fluorescence. The plates were incubated overnight at 37°C. Ampicillin resistant transformants
15 were sprayed with a 1 mM IPTG solution to induce luciferase expression. After allowing time for the cells to express the luciferase, the surface of the plates were sprayed with a solution containing 20 mM coelenterazine and colonies emitting blue light were visualized using a blue bandwidth filter.

Plasmid DNA was isolated from cultures of bioluminescent transformants
20 and the nucleotide sequence of a cDNA insert of a positive clone was determined. The nucleotide sequence of DNA encoding a full-length *Gaussia* luciferase and the amino acid sequence are set forth in SEQ ID Nos. 19 and 20. The cDNA fragment encoding the *Gaussia* luciferase is 765 nt in length, including 5' non-coding region, a 455 nt open
25 reading frame encoding a 185 amino acid polypeptide, and 3' non-coding sequence.

EXAMPLE 9

CLONING ADDITIONAL LUCIFERASE AND GFP PROTEINS

Using the methods described in the preceding examples, a nucleic acid
30 encoding a GFP for a *Ptilosarcus* (a sea pen obtained from the Sea of Cortez) species, and a luciferase from a *Pleuromamma* (a copepod) species was obtained. The sequences are set forth in SEQ ID NOs. 28-32. The

-159-

Pleuromamma luciferase was cloned from a mixed copepod library of *Gaussia* and *Pleuromamma* ("giant" calenoid copepods, ~6 mm and ~3 mm, respectively). The *Pleuromamma* luciferase was identified by its greener *in vivo* emission.

- 5 Emission spectra and pH and salt curves for the encoded proteins are provided in FIGURES 4-6 and 8-10.

EXAMPLE 10

- Gaussia*, *Renilla mulleri* and *Pleuromamma* luciferases and *Renilla mulleri* and *Ptilosarcus* GFPs have been cloned. Various nucleic acid constructs and
10 plasmids containing nucleic acid encoding these proteins have been prepared and have been used for expression of the encoded proteins for use in diagnostics, in analytical procedures, and in the novelty items as described herein.

Constructs

- 15 A. *Renilla mulleri* luciferase and GFP-encoding constructs and plasmids

- The host plasmid is a pBluescript SK(-) phagemid (Stratagene). The construct shown is one that was isolated by the functional screening of a large population of phagemids derived from mass excision of an amplified Lambda ZAP cDNA library (Stratagene). Each of the cloned bioluminescent genes
20 described herein were isolated in a similar phagemid as an insert between the EcoRI and XhoI sites of the multiple cloning site (MCS). Each insert includes DNA that encompasses the entire coding region (CDS) of the functional protein, as well as a variable number of nucleotides 5' and 3' of the coding region. In addition to the amino acids of the native protein, the polypeptides expressed in
25 the functionally screened isolates (here, the *lacZ-Renilla mulleri* luciferase cDNA fusion CDS or the *lacZ-Renilla mulleri* GFP cDNA fusion CDS) can contain additional N-terminal residues.

1) *Renilla mulleri* luciferase in pBluescript SK-(r) (4147 bp)

- A *lacZ-Renilla mulleri* luciferase coding domain sequence (CDS) fusion
30 was cloned into the pBluescript SK-(r) (Stratagene). This well known commercially available vector contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Ampicillin), a phage replication

-160-

origin (f1 origin), T3 primer sequence, T3 20-mer sequence, NIH oligo 0495 sequence, T3 promoter, SK primer sequence, T7 promoter, KS primer sequence, T7 primer sequence, T7 22-mer sequence, NIH oligo 0436 sequence, phage-plasmid PCR1 sequence, phage-plasmid PCR2 sequence, phage-plasmid PCR2(b) sequence and various restriction cloning sites. Expression of the lacZ-*Renilla mulleri* luciferase fusion protein is under the control of lacZ promoter.

2) *Renilla mulleri* luciferase in pBluescript SK (4147 bp)

A lacZ-*Renilla mulleri* luciferase coding domain sequence (CDS) fusion was cloned into the pBluescript SK-(r) (Stratagene), which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Ampicillin), a phage replication origin (f1 origin), T3 primer sequence, T3 promoter, SK primer sequence, T7 promoter, KS primer sequence, T7 primer sequence and various restriction cloning sites. Expression of the lacZ-*Renilla mulleri* luciferase fusion protein is under the control of lacZ promoter.

15 B. Plasmids for expression of native *Renilla mulleri* GFP and luciferase in mammalian cells

The *Renilla mulleri* GFP or luciferase coding region was amplified by nucleic acid amplification (PCR), respectively appending an EcoRI site and a XhoI site immediately 5' and 3' to the coding sequence. The PCR product was inserted between the *EcoRI-XhoI* sites in the polylinker of pcDNA3.1(+) (Invitrogen), and transformed into bacteria (*e.g.*, XLI-Blue strain, Stratagene) for the purpose of producing large quantities of plasmid DNA. These plasmids contain the CMV promoter (Pcmv) to drive expression in mammalian cells.

1) *Renilla mulleri* GFP in pcDNA3.1(+) (6122 bp)

A *Renilla mulleri* GFP CDS was cloned into the pcDNA3.1(+) (Invitrogen, San Diego), which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Amp), a mammalian selectable marker (Neo), a phage replication origin (f1 origin), a viral replication origin (SV40 origin) and various restriction cloning sites. Expression of the *Renilla mulleri* GFP is under the control of CMV promoter.

-161-

2) *Renilla mulleri* luciferase in pcDNA3.1(+) (6341 bp)

A *Renilla mulleri* luciferase CDS was cloned into the pcDNA3.1(+) (Invitrogen, San Diego) which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Amp), a mammalian selectable
5 marker (Neo), a phage replication origin (f1 origin), a viral replication origin (SV40 origin) and various restriction cloning sites. Expression of the *Renilla mulleri* luciferase is under the control of CMV promoter.

4. Plasmids used to express native *Renilla mulleri* GFP and luciferase in yeast cells

10 The *Renilla mulleri* GFP or luciferase was PCR amplified and inserted between the polylinker *EcoRI-XhoI* sites of pYES2 (Invitrogen). These plasmids are designed for galactose-inducible expression in yeast under regulation of the GAL1 promoter.

1) *Renilla mulleri* GFP in pYES2 (6547 bp)

15 A *Renilla mulleri* GFP CDS was cloned into the yeast expression plasmid pYES2 (Invitrogen, San Diego), which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Amp), a yeast replication origin (2 micron origin), a yeast selectable marker (URA3), a phage replication origin (f1 origin) and various restriction cloning sites. Expression of the *Renilla*
20 *mulleri* GFP is under the control of yeast GAL1. This vector is designed for expression in *Saccharomyces cerevisiae* cells.

2) *Renilla mulleri* luciferase in pYES2 (6766 bp)

A *Renilla mulleri* luciferase CDS was cloned into the pYES2 (Invitrogen) which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial
25 selectable marker (Amp), a yeast replication origin (2 micron origin), a yeast selectable marker (URA3), a phage replication origin (f1 origin) and various restriction cloning sites. Expression of the *Renilla mulleri* luciferase is under the control of yeast GAL1.

30 D. Plasmids used to express native *Renilla mulleri* GFP or luciferase in bacterial cells

Using the pET-34 CBD-*Renilla mulleri* luciferase or pET-34 CBD-*Renilla mulleri* GFP plasmid as template, high fidelity inverse PCR was used to

-162-

precisely delete the CBD and all other coding sequences 5' to the native *Renilla mulleri* luciferase or GFP start codon. The plasmids were recircularized and reintroduced into BL21 (DE3) cells (Novagen, Madison, WI). These plasmids are designed to express large quantities of native-length polypeptide upon induction with IPTG. Dependent on the nature of the expressed polypeptide, the protein can fold properly and reside in a functional form in the cytosol or be released into the culture medium. When expressed in this manner, significant functional activity is observed for all bioluminescent proteins described herein. The *Gaussia* luciferase is released into the culture medium.

10 **1) Native *Ptilosarcus* GFP in pET-34 (6014 bp)**

A *Ptilosarcus* GFP CDS was cloned into the pET-34 (Novagen) which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Kan), a phage replication origin (f1 origin), lacI sequence, T7 promoter, lac operator, ribosome binding sequence (rbs), LIC (ligation independent cloning) site, 3' LIC overlap sequence, His tag CDS, His tag sequence, T7 terminator and various restriction cloning sites. Expression of the *Ptilosarcus* GFP is under the joint control of lac operator and T7 promoter, which is inducible by IPTG.

20 **2) Native *Renilla mulleri* GFP in pET-34 (6014 bp)**

A *Renilla mulleri* GFP CDS was cloned into the pET-34 (Novagen) which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Kan), a phage replication origin (f1 origin), lacI sequence, T7 promoter, lac operator, rbs, LIC site, 3' LIC overlap sequence, His tag CDS, His tag sequence, T7 terminator and various restriction cloning sites. Expression of the *Renilla mulleri* GFP is under the joint control of lac operator and T7 promoter, which is inducible by IPTG.

25 **3) Native *Gaussia* luciferase in pET-34 (5855 bp)**

A *Gaussia* luciferase CDS was cloned into the pET-34 (Novagen) which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Kan), a phage replication origin (f1 origin), lacI sequence, T7 promoter, lac operator, rbs, LIC site, 3' LIC overlap sequence, His tag CDS, His tag sequence, T7 terminator and various restriction cloning sites. Expression of

-163-

the *Gaussia* luciferase is under the joint control of lac operator and T7 promoter, which is inducible by IPTG.

4) Native *Pleuromamma* luciferase in pET-34 (5894 bp)

A *Pleuromamma* luciferase CDS was cloned into the pET-34 (Novagen) which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Kan), a phage replication origin (f1 origin), lacI sequence, T7 promoter, lac operator, rbs, LIC site, 3' LIC overlap sequence, His tag CDS, His tag sequence, T7 terminator and various restriction cloning sites. Expression of the *Pleuromamma* luciferase is under the joint control of lac operator and T7 promoter, which is inducible by IPTG.

5) Native *Renilla mulleri* luciferase in pET-34 (6233 bp)

A *Renilla mulleri* luciferase CDS was cloned into the pET-34 (Novagen) which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Kan), a phage replication origin (f1 origin), lacI sequence, T7 promoter, lac operator, rbs, LIC site, 3' LIC overlap sequence, His tag CDS, His tag sequence, T7 terminator and various restriction cloning sites. Expression of the *Renilla mulleri* luciferase is under the joint control of lac operator and T7 promoter, which is inducible by IPTG.

5. Plasmids used to purify a cellulose binding domain-*Renilla mulleri* luciferase or GFP fusion protein from bacterial cells

The coding region of the *Renilla mulleri* luciferase or GFP was amplified with a high fidelity polymerase (*Pfu turbo*, Stratagene) using the cloned pBluescript phagemid as template, and inserted using a ligation independent cloning (LIC) site into the pET-34 LIC vector (Novagen). The resulting cellulose binding domain (CBD)-luciferase or CBD-GFP fusion protein can be expressed at high levels in BLI(DE3) cells (Novagen) after induction with IPTG. Due to the nature of CBD-clos, the major portion of the expressed protein will reside in insoluble inclusion bodies. Inclusion bodies can be isolated in semi-pure state and functional CBD-fusion proteins can be recovered by renaturation. Inclusion of the thrombin and enterokinase (EK) cleavage sites in the fusion protein permits isolation of highly purified native or near-native proteins for the purposes of rigorous analysis.

-164-

1) CBD-*Renilla mulleri* luciferase in pET-34 (6824 bp)

A CBD-*Renilla mulleri* luciferase CDS was cloned into the pET-34 (Novagen), which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Kan), a phage replication origin (f1 origin), lacI sequence, T7 promoter, lac operator, rbs, LIC site, 3' LIC overlap sequence, His tag CDS, His tag sequence, T7 terminator, thrombin cleavage site, the RNase-S-peptide tag (S tag) CDS sequence, EK cleavage site and various restriction cloning sites. Expression of the CBD-*Renilla mulleri* luciferase is under the joint control of lac operator and T7 promoter, which is inducible by IPTG.

2) CBD-*Gaussia* luciferase in pET-34 (6446 bp)

A CBD-*Gaussia* luciferase CDS was cloned into the pET-34 (Novagen), which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Kan), a phage replication origin (f1 origin), lacI sequence, T7 promoter, lac operator, rbs, LIC site, 3' LIC overlap sequence, His tag CDS, His tag sequence, T7 terminator, thrombin cleavage site, S tag CDS sequence, EK cleavage site and various restriction cloning sites. The expression of the CBD-*Gaussia* luciferase is under the joint control of the lac operator and T7 promoter, which is inducible by IPTG.

3) CBD-*Pleuromamma* luciferase in pET-34 (6485 bp)

A CBD-*Pleuromamma* luciferase CDS was cloned into the pET-34 (Novagen), which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Kan), a phage replication origin (f1 origin), lacI sequence, T7 promoter, lac operator, rbs sequence, LIC site, 3' LIC overlap sequence, His tag CDS, His tag sequence, T7 terminator, thrombin cleavage site, S tag CDS sequence, EK cleavage site and various restriction cloning sites. Expression of the CBD-*Pleuromamma* luciferase is under the joint control of lac operator and T7 promoter, which is inducible by IPTG.

4) CBD-*Ptilosarcus* GFP in pET-34 (6605 bp)

A CBD-*Ptilosarcus* GFP CDS was cloned into the pET-34 (Novagen) which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Kan), a phage replication origin (f1 origin), lacI sequence, T7 promoter, lac operator, rbs sequence, LIC site, 3' LIC overlap sequence, His tag

-165-

CDS, His tag sequence, T7 terminator, thrombin cleavage site, S tag CDS sequence, EK cleavage site and various restriction cloning sites. Expression of the CBD-*Ptilosarcus* GFP is under the joint control of lac operator and T7 promoter, which is inducible by IPTG.

5 **5) CBD-*Renilla mulleri* GFP in pET-34 (6605 bp)**

A CBD-*Renilla mulleri* GFP CDS was cloned into the pET-34 (Novagen) which contains, *inter alia*, a bacterial replication origin (ColE1 origin), a bacterial selectable marker (Kan), a phage replication origin (f1 origin), lacI sequence, T7 promoter, lac operator, rbs sequence, LIC site, 3' LIC overlap sequence, His tag
10 CDS, His tag sequence, T7 terminator, thrombin cleavage site, S tag CDS sequence, EK cleavage site and various restriction cloning sites. The expression of the CBD-*Renilla mulleri* GFP is under the joint control of the lac operator and T7 promoter, which is inducible by IPTG.

F. Plasmid for expressing *Renilla mulleri* luciferase-GFP fusion protein

15 As depicted in Figure 7, *Renilla mulleri* GFP was inserted into the ligation independent cloning site of the pET-34 vector (Novagen). The cellulose binding domain (CBD) that is normally present in pET-34 was deleted using inverse PCR. To facilitate optimization for analytical uses of the resulting fusion protein (such as for BRET), additional restriction sites (not shown) have been introduced into
20 the linker region, permitting insertion of desired linking and target proteins or moieties. Using two of these sites, the *Renilla mulleri* luciferase CDS was inserted in the standard position of the CBD. In this plasmid, or in similar constructs that retain the CBD, near-native and native GFP can be cleaved from the fusion protein by treatment with thrombin or enterokinase (EK), respectively.
25 The RNase S-peptide tag (S tag CDS) facilitates immunoaffinity purification of the GFP or the fusion protein, and allows quantitation of these proteins in crude extracts using a commercial RNase assay (Novagen). If the luciferase is separately fused to the S-tag in a second separate plasmid, association or co-expression of the S-protein/luciferase and S-protein/GFP via the RNase domain
30 creates a test system for intermolecular BRET.

G. Functional expression of *Renilla mulleri* GFP in mammalian cells

-166-

HeLa cells were transfected with the plasmid pcDNA3.1(+) containing *Renilla mulleri* GFP under the control of a CMV promoter (construct No. B(1) as described above). HeLa cells were burst transfected with 1.5 micrograms of pcDNA3.1 per plate using the LipofectAMINE plus kit (GIBCO).

- 5 When HeLa cells were burst transfected with 30 micrograms of pcDNA-*Renilla mulleri* GFP DNA and grown at 37°C for 8 hours, a sub-population of cells were observed to be highly fluorescent. The fluorescence was localized to pairs of cells that were undergoing, or apparently had recently undergone, a single round of cell-division. This result indicates that native *Renilla mulleri* GFP
- 10 can be expressed, folded and complexed properly with the chromophore, and retain its function to produce green fluorescence in mammalian cells. This is in contrast to the *Aequorea* GFP, which folds inefficiently into a functional form under the physiological conditions.

- 15 Filters (470/40 excitation filter, 495 excitation dichroic filter and 525/50 emission filter) used in visualization of *R. mulleri* GFP fluorescence in HeLa cells were the Endo GFP filter set sold by Chroma.

- 20 Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.

-167-

Summary of the Sequence Listing

1. SEQ ID NO. 1 *Renilla reniformis* Luciferase [U.S. Patent No. 5,418,155]
2. SEQ ID NO. 2 *Cypridina hilgendorffii* luciferase [EP 0 387 355]
3. SEQ ID NO. 3 Modified *Luciola cruciata* Luciferase [firefly; U.S. Patent
5 No. 4,968,613]
4. SEQ ID NO. 4 *Vargula (Cypridina)* luciferase [Thompson et al. (1989)
Proc. Natl. Acad. Sci. U.S.A. 86:6567-6571 and from JP 3-30678
Osaka
5. SEQ ID NO. 5 Apoaequorin-encoding gene [U S. Patent No. 5,093,240,
10 PAQ440]
6. SEQ ID NO. 6 Encoded *Aequorin* AEQ1 [Prasher et al. (1987) "Sequence
Comparisons of CDNAS Encoding for Aequorin Isotypes," Biochemistry
26:1326-1332]
7. SEQ ID NO. 7 Encoded *Aequorin* AEQ2 [Prasher et al. (1987)]
- 15 8. SEQ ID NO. 8 Encoded *Aequorin* AEQ3 [Prasher et al. (1987)]
9. SEQ ID NO. 9 *Aequorin* photoprotein [Charbonneau et al. (1985)
"Amino Acid Sequence of the Calcium-Dependent Photoprotein
Aequorin," Biochemistry 24:6762-6771]
10. SEQ ID NO. 10 *Aequorin* mutant with increased bioluminescence activity
20 [U.S. Patent No. 5,360,728; Asp 124 changed to Ser]
11. SEQ ID NO. 11 *Aequorin* mutant with increased bioluminescence activity
[U.S. Patent No. 5,360,728; Glu 135 changed to Ser]
12. SEQ ID NO. 12 *Aequorin* mutant with increased bioluminescence
activity [U.S. Patent No. 5,360,728 Gly 129 changed to Ala]
- 25 13. SEQ ID NO. 13 Encoded apoaequorin [sold by Sealite, Sciences, Bogart,
GA as AQUALITE®, when reconstituted to form aequorin]
14. SEQ ID NO. 14 *Vibrio fischeri* Flavin reductase [U.S. Patent No.
5,484,723]
15. SEQ ID NO. 15 nucleic acid encoding *Renilla mulleri* green fluorescent
30 protein (GFP)
16. SEQ ID NO. 16 Encoded *Renilla mulleri* green fluorescent protein (GFP)
17. SEQ ID NO. 17 nucleic acid encoding *Renilla mulleri* luciferase

-168-

18. SEQ ID NO. 18 Encoded *Renilla mulleri* luciferase
19. SEQ ID NO. 19 nucleic acid encoding *Gausssia* luciferase
20. SEQ ID NO. 20 Encoded *Gausssia* luciferase
21. SEQ ID NO. 21 nucleic acid encoding a *Gaussia* luciferase fusion protein
- 5 22. SEQ ID NO. 22 Encoded *Gaussia* luciferase fusion protein
23. SEQ ID NO. 23 *Renilla reniformis* GFP peptide corresponding to amino acid sequences near the amino-terminus of *R. mulleri* GFP
24. SEQ ID NO. 24 *Renilla reniformis* GFP peptide corresponding to amino acid sequences near the amino-terminus of *R. mulleri* GFP
- 10 25. SEQ ID NO. 25 *Renilla reniformis* GFP peptide corresponding to amino acid sequences near the middle of *R. mulleri* GFP
26. SEQ ID NO. 26 *Renilla reniformis* GFP peptide corresponding to amino acid sequences near the middle of *R. mulleri* GFP
27. SEQ ID NO. 27 *Renilla reniformis* GFP peptide corresponding to amino acid sequences near the carboxy-terminus of *R. mulleri* GFP
- 15 28. SEQ ID NO. 28 Pleuromamma luciferase\insert 861 bp
29. SEQ ID No. 29 encoded Pleuromamma luciferase 198 aa
30. SEQ ID NO:30 Ptilosarcus GFP\insertA\1104 bp
31. SEQ ID NO:31 Ptilosarcus GFP\insertB\1279 bp
- 20 32. SEQ ID NO:32 Ptilosarcus GFP 238 aa

-169-

WHAT IS CLAIMED IS:

1. An isolated nucleic acid fragment, comprising a sequence of nucleotides encoding a *Renilla mulleri* luciferase, a *Gaussia* luciferase or a *Pleuromamma* luciferase.
- 5 2. The isolated nucleic acid fragment of claim 1, wherein the *Gaussia* is a member of the species of *princeps*.
3. The isolated nucleic acid fragment of claim 1, wherein the nucleic acid is DNA.
4. The isolated nucleic acid fragment of claim 1, wherein the nucleic acid is RNA.
- 10 5. An isolated nucleic acid fragment of claim 1, comprising the sequence of nucleotides selected from the group consisting of
a sequence of nucleotides set forth in SEQ ID No. 17, SEQ ID No. 19, or SEQ ID No. 28;
15 a sequence of nucleotides encoding the amino acid sequence set forth in SEQ ID No. 18, SEQ ID No. 20 or SEQ ID No. 29; and
a sequence of nucleotides that hybridizes under high stringency to the sequence of nucleotides set forth in SEQ ID No. 17, SEQ ID No. 19 or SEQ ID No. 28.
- 20 6. A nucleic acid probe or primer, comprising at least 14 contiguous nucleotides selected from the sequence of nucleotides set forth in the coding portion of SEQ ID Nos. 17, 19 or 28.
7. The nucleic acid probe or primer of claim 6, comprising at least 16 contiguous nucleotides selected from the sequence of nucleotides set forth
25 in the coding portion of SEQ ID Nos. 17, 19 or 28.
8. The nucleic acid probe or primer of claim 6, comprising at least 30 contiguous nucleotides selected from the sequence of nucleotides set forth in the coding portion of SEQ ID Nos. 17, 19 or 28.
9. A plasmid, comprising the nucleic acid fragment of claim 1.
- 30 10. The plasmid of claim 9 that is an expression vector.
11. The plasmid of claim 10, comprising a sequence of nucleotides encoding:

-170-

a promoter region;

a *Gaussia*, *Pleuromamma* or *Renilla mulleri* luciferase; and

a selectable marker;

wherein the sequence of nucleotides encoding the luciferase is operatively
5 linked to the promoter, whereby the luciferase is expressed.

12. The plasmid of claim 10, further comprising a sequence of nucleotides encoding a green fluorescent protein (GFP).

13. A recombinant host cell, comprising the plasmid of claim 10.

14. The cell of claim 13, wherein the cell is selected from the group
10 consisting of a bacterial cell, a yeast cell, a fungal cell, a plant cell, an insect cell and an animal cell.

15. A method for producing a *Gaussia*, *Renilla mulleri* or *Pleuromamma* luciferase protein, comprising growing the recombinant host cell of claim 13, wherein the luciferase protein is expressed by the cell, and
15 recovering the expressed luciferase protein.

16. An isolated substantially purified *Gaussia*, *Renilla mulleri* or *Pleuromamma* luciferase protein.

17. The isolated protein of claim 16, wherein the protein has the sequence of amino acids set forth in SEQ ID Nos. 18, 20 or 29.

20 18. A combination, comprising:
an article of manufacture; and
a bioluminescence generating system, whereby the combination is a novelty item, wherein the bioluminescence generating system comprises a luciferase encoded by the nucleic acid of claim 1.

25 19. The combination of claim 18, further comprising a luciferin.

20. The combination of claim 18, further comprising a green fluorescent protein (GFP).

21. The combination of claim 20, wherein the GFP is a *Renilla* GFP or a *Ptilocarpus* GFP.

30 22. The combination of claim 18, wherein the article of manufacture is selected from among toys, cosmetics, fountains, personal care items, fairy

-171-

dust, beverages, soft drinks, foods, textile products, bubbles, balloons, personal items, dentifrices, soaps, body paints, bubble bath, ink and paper products.

23. The combination of claim 22 that is a toy gun.
24. The combination of claim 22 that is a food.
- 5 25. The combination of claim 22 that is a beverage.
26. The combination of claim 22 that is a cosmetic.
27. The combination of claim 18, wherein the article of manufacture is selected from among squirt guns, pellet guns, finger paints, foot bags, greeting cards, slimy play material, clothing, bubble making toys, bath powders,
10 cosmetics, body lotions, gels, body powders, body creams, toothpastes, mouthwashes, soaps, body paints, bubble bath, inks, wrapping paper, gelatins, icings, frostings, greeting cards, beer, wine, champagne, soft drinks, ice cubes, ice, dry ice and fountains.
28. The combination of claim 27 that is a toy gun.
- 15 29. The combination of claim 27 that is a food.
30. The combination of claim 27 that is a beverage.
31. The combination of claim 27 that is a cosmetic.
32. An isolated nucleic acid fragment, comprising a sequence of nucleotides encoding a *Renilla* green fluorescent protein (GFP) or a *Ptilocarpus*
20 green fluorescent protein.
33. The isolated nucleic acid fragment of claim 32, wherein the *Renilla* species is selected from the group consisting of *Renilla reniformis*, *Renilla kollokeri* and *Renilla mulleri*.
34. The isolated nucleic acid fragment of claim 32, wherein the
25 nucleic acid is DNA.
35. The isolated nucleic acid fragment of claim 32, wherein the nucleic acid is RNA.
36. An isolated nucleic acid fragment encoding a green fluorescent protein (GFP), comprising the sequence of nucleotides selected from the group
30 consisting of
a sequence of nucleotides set forth in SEQ ID No. 15, SEQ ID No. 30 or SEQ ID No. 31;

-172-

a sequence of nucleotides encoding the amino acid sequence set forth in SEQ ID No. 16 or SEQ ID No. 32; and

a sequence of nucleotides that hybridizes under high stringency to the sequence of nucleotides set forth in SEQ ID No. 15, SEQ ID No. 30 or SEQ ID No. 31.

37. A nucleic acid probe or primer, comprising at least 14 contiguous nucleotides selected from the sequence of nucleotides set forth in SEQ ID No. 15, SEQ ID No. 30 or SEQ ID No. 31.

38. The probe or primer of claim 37, comprising at least 16 contiguous nucleotides selected from the sequence of nucleotides set forth in SEQ ID No. 15, SEQ ID No. 30 or SEQ ID No. 31.

39. The probe or primer of claim 37, comprising at least 30 contiguous nucleotides selected from the sequence of nucleotides set forth in SEQ ID No. 15, SEQ ID No. 30 or SEQ ID No. 31.

40. A plasmid, comprising the sequence of nucleotides of claim 32.

41. An expression vector, comprising:

the plasmid of claim 40;

a promoter element;

a multiple cloning site for the introduction of nucleic acid; and

a selectable marker;

wherein the nucleic acid encoding the multiple cloning site is positioned between nucleic acids encoding the promoter element and the green fluorescent protein and wherein the nucleic acid encoding the green fluorescent protein is operatively linked to the promoter element.

42. The plasmid of claim 40, further comprising a sequence of nucleotides encoding:

a promoter element;

a selectable marker;

wherein, the sequence of nucleotides encoding the green fluorescent protein is operatively linked to the promoter element, whereby the green fluorescent protein is expressed.

-173-

43. The plasmid of claim 42, further comprising a sequence of nucleotides encoding a luciferase.

44. A recombinant host cell, comprising the plasmid of claim 40.

45. The cell of claim 44, wherein the cell is selected from the group consisting of a bacterial cell, a yeast cell, a fungal cell, a plant cell, an insect cell and an animal cell.

46. An isolated substantially purified *Renilla mulleri* green fluorescent protein (GFP) or a *Ptilosarcus* GFP.

47. A composition, comprising the green fluorescent protein of claim 46.

48. The composition of claim 47, further comprising at least one component of a bioluminescence generating system.

49. The composition of claim 48, wherein the bioluminescence generating system is selected from those isolated from: an insect system, a coelenterate system, a ctenophore system, a bacterial system, a mollusk system, a crustacea system, a fish system, an annelid system, and an earthworm system.

50. The composition of claim 48, wherein the bioluminescence generating system is selected from those isolated from: fireflies, *Mnemiopsis*, *Beroe ovata*, *Aequorea*, *Obelia*, *Vargula*, *Pelagia*, *Renilla*, *Pholas*, *Aristostomias*, *Pachystomias*, *Porichthys*, *Cypridina*, *Aristostomias*, such *Pachystomias*, *Malacosteus*, *Gonadostomias*, *Gaussia*, *Watensia*, *Halisturia*, Vampire squid, *Glyphus*, Mycotophids, *Vinciguerria*, *Howella*, *Florenciella*, *Chaudiodus*, *Melanocostus*, Sea Pens, *Chiroteuthis*, *Eucleoteuthis*, *Onychoteuthis*, *Watasenia*, cuttlefish, *Sepiolina*, *Oplophorus*, *Acanthophyra*, *Sergestes*, *Gnathophausia*, *Argyropelecus*, *Yarella*, *Diaphus*, *Gonadostomias* and *Neoscopelus*.

51. The composition of claim 50, wherein the bioluminescence generating system is selected from those isolated from *Aequorea*, *Obelia*, *Vargula* and *Renilla*.

52. A combination, comprising:
an article of manufacture; and

-174-

a *Renilla* or *Ptilosarcus* green fluorescent protein (GFP).

53. The combination of claim 52, further comprising
at least one component of a bioluminescence generating system,
whereby the combination is a novelty item.

5 54. The combination of claim 53, wherein the combination comprises
a luciferase.

55. The combination of claim 53, wherein the combination comprises
a luciferin.

10 56. The combination of claim 53, wherein the combination comprises
a luciferin and a luciferase.

57. The combination of claim 52, wherein the article of manufacture
is selected from among toys, fountains, personal care items, cosmetics, fairy
dust, beverages, soft drinks, foods, textile products, bubbles, balloons, personal
items, dentifrices, soaps, body paints, bubble bath, ink and paper products.

15 58. The combination of claim 57 that is a toy gun.

59. The combination of claim 57 that is a food.

60. The combination of claim 57 that is a cosmetic.

61. The combination of claim 57 that is a beverage.

20 62. The combination of claim 52, wherein the article of manufacture
is selected from among squirt guns, pellet guns, finger paints, foot bags, slimy
play material, clothing, bubble making toys, bath powders, body lotions, gels,
body powders, body creams, toothpastes, mouthwashes, soaps, body paints,
bubble bath, inks, wrapping paper, gelatins, icings, frostings, beer, wine,
champagne, soft drinks, ice cubes, ice, dry ice and fountains.

25 63. The combination of claim 62 that is a toy gun.

64. The combination of claim 62 that is a food.

65. The combination of claim 62 that is a cosmetic.

66. The combination of claim 62 that is a beverage.

30 67. An antibody that immuno-specifically binds a *Renilla mulleri*
luciferase, a *Gaussia* luciferase or a *Pleuromamma* luciferase, or a fragment or
derivative of said antibody containing the binding domain thereof.

68. The antibody of claim 67 which is a monoclonal antibody.

-175-

69. An antibody that immuno-specifically binds a *Renilla* green fluorescent protein (GFP) or a *Ptilocarpus* GFP, or a fragment or derivative of said antibody containing the binding domain thereof.

70. The antibody of claim 69, wherein the *Renilla* GFP is a *Renilla*
5 *reniformis*, *Renilla kollokeri* or *Renilla mulleri* GFP.

71. The antibody of claim 69 which is a monoclonal antibody.

72. A nucleic acid construct, comprising a nucleotide sequence encoding a luciferase and a nucleotide sequence encoding a green fluorescent protein (GFP).

10 73. The nucleic acid construct of claim 72, wherein the luciferase is a *Renilla mulleri* luciferase, a *Gaussia* luciferase or a *Pleuromamma* luciferase.

74. The nucleic acid construct of claim 73, wherein the *Gaussia* luciferase is a *Gaussia princeps* luciferase.

15 75. The nucleic acid construct of claim 72, wherein the luciferase is encoded by:

a sequence of nucleotides set forth in SEQ ID No. 17, SEQ ID No. 19, or SEQ ID No. 28;

a sequence of nucleotides encoding the amino acid sequence set forth in SEQ ID No. 18, SEQ ID No. 20 or SEQ ID No. 29; and

20 a sequence of nucleotides that hybridizes under high stringency to the sequence of nucleotides set forth in SEQ ID No. 17, SEQ ID No. 19 or SEQ ID No. 28.

76. The nucleic acid construct of claim 72, wherein the GFP is a *Renilla* green fluorescent protein (GFP) or a *Ptilocarpus* GFP.

25 77. The nucleic acid construct of claim 76, wherein the *Renilla* GFP is a *Renilla reniformis*, *Renilla kollokeri* or *Renilla mulleri* GFP.

78. The nucleic acid construct of claim 72, wherein the GFP is encoded by:

30 a sequence of nucleotides set forth in SEQ ID No. 15, SEQ ID No. 30 or SEQ ID No. 31;

a sequence of nucleotides encoding the amino acid sequence set forth in SEQ ID No. 16 or SEQ ID No. 32; and

-176-

a sequence of nucleotides that hybridizes under high stringency to the sequence of nucleotides set forth in SEQ ID No. 15, SEQ ID No. 30 or SEQ ID No. 31.

79. The nucleic acid construct of claim 72, wherein the luciferase and
5 GFP are from *Renilla*.

80. The nucleic acid construct of claim 79, wherein the *Renilla* luciferase and GFP are from *Renilla mulleri*.

81. The nucleic acid construct of claim 72, wherein the nucleotide sequence encoding the luciferase and GFP are linked contiguously.

10 82. The nucleic acid construct of claim 72 which is DNA.

83. The nucleic acid construct of claim 72 which is RNA.

84. A plasmid, comprising the nucleic acid construct of claim 72.

85. The plasmid of claim 84, further comprising a sequence of nucleotides encoding:

15 a promoter element;

a selectable marker;

wherein, the sequence of nucleotides encoding the luciferase and GFP is operatively linked to the promoter element, whereby a fusion protein of the luciferase and GFP is expressed.

20 86. A recombinant host cell, comprising the plasmid of claim 84.

87. The cell of claim 86, wherein the cell is selected from the group consisting of a bacterial cell, a yeast cell, a fungal cell, a plant cell, an insect cell and an animal cell.

88. An isolated substantially purified luciferase and GFP fusion
25 protein.

89. The fusion protein of claim 88, wherein the luciferase and GFP are from *Renilla*.

90. The fusion protein of claim 89, wherein the *Renilla* luciferase and GFP are from *Renilla mulleri*.

30 91. A composition, comprising the fusion protein of claim 88.

92. The composition of claim 91, further comprising at least one component of a bioluminescence generating system.

-177-

93. The composition of claim 92, wherein the component of the bioluminescence generating system is luciferin.

94. The nucleic acid construct of claim 72, wherein the nucleotide sequence encoding the luciferase and GFP are not contiguous.

5 95. The nucleic acid construct of claim 94, comprising a sequence of nucleotides that encodes a ligand binding domain of a target protein.

96. A nucleic acid probe or primer, comprising at least 14 nucleotides, preferably 16 to 30 nucleotides, that are based on amino acids 51 to 68, 82 to 98 and 198 to 208 set forth in SEQ ID No. 16, amino acid sequence set forth in
10 SEQ ID No. 24, amino acids 9-20 set forth in SEQ ID No. 25 or amino acids 39-53 set forth in SEQ ID No. 27.

97. The probe or primer of claim 96, comprising a sequence of nucleic acids in SEQ ID No.15 that encodes the sequence of amino acids.

98. A method of isolating nucleic acid that encodes a *Renilla* green
15 fluorescent protein (GFP), comprising:

screening a *Renilla* nucleic acid library with a probe or plurality of probes of claim 96; and

identifying and isolating nucleic acid that encodes a GFP.

99. A method of isolating nucleic acid that encodes a *Renilla* green
20 fluorescent protein (GFP), comprising:

amplifying nucleic acid in a *Renilla* nucleic acid library with a primer or plurality of primers of claim 96; and

isolating the amplified nucleic acids, whereby nucleic acid encoding a GFP is identified and isolated.

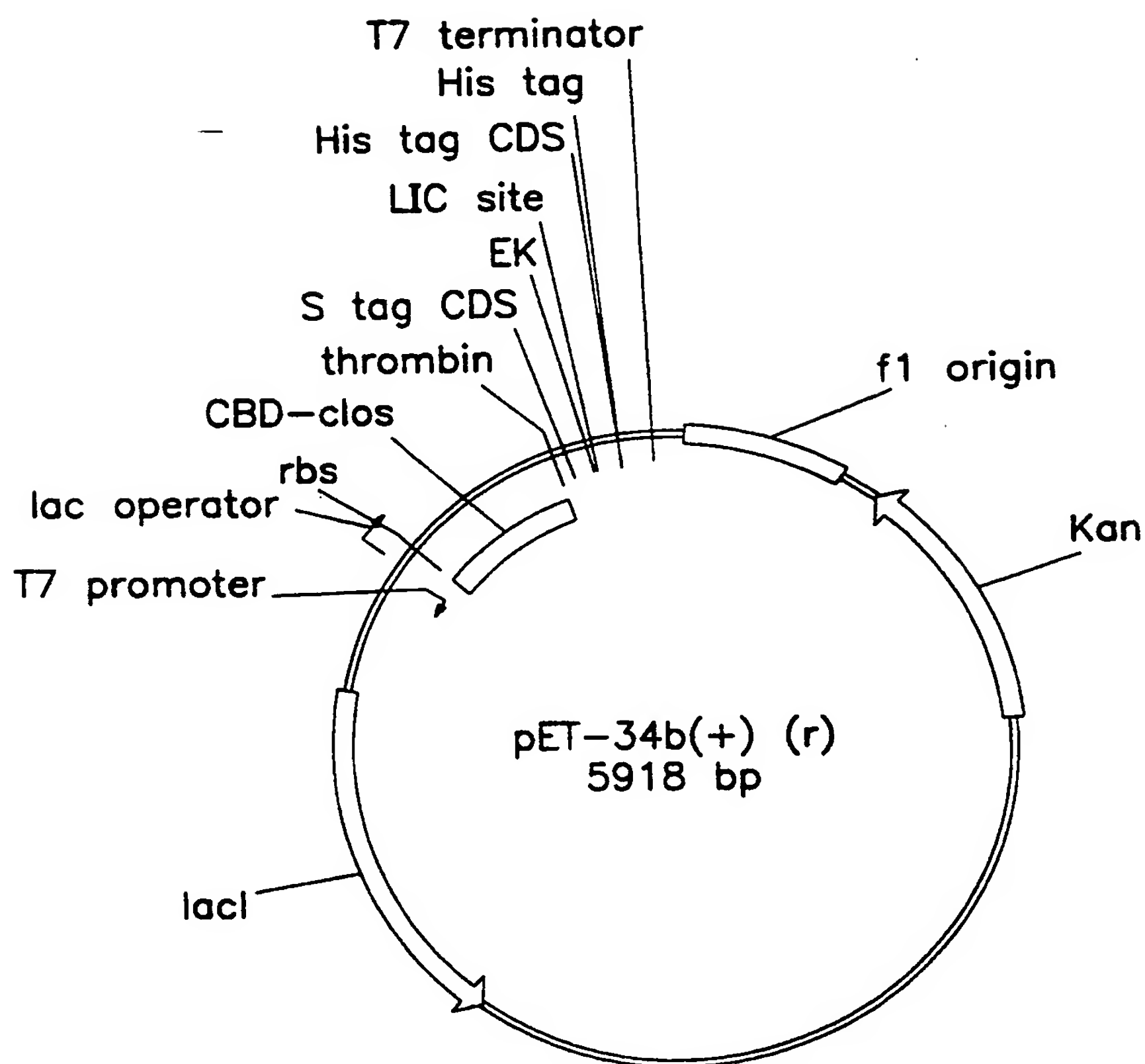
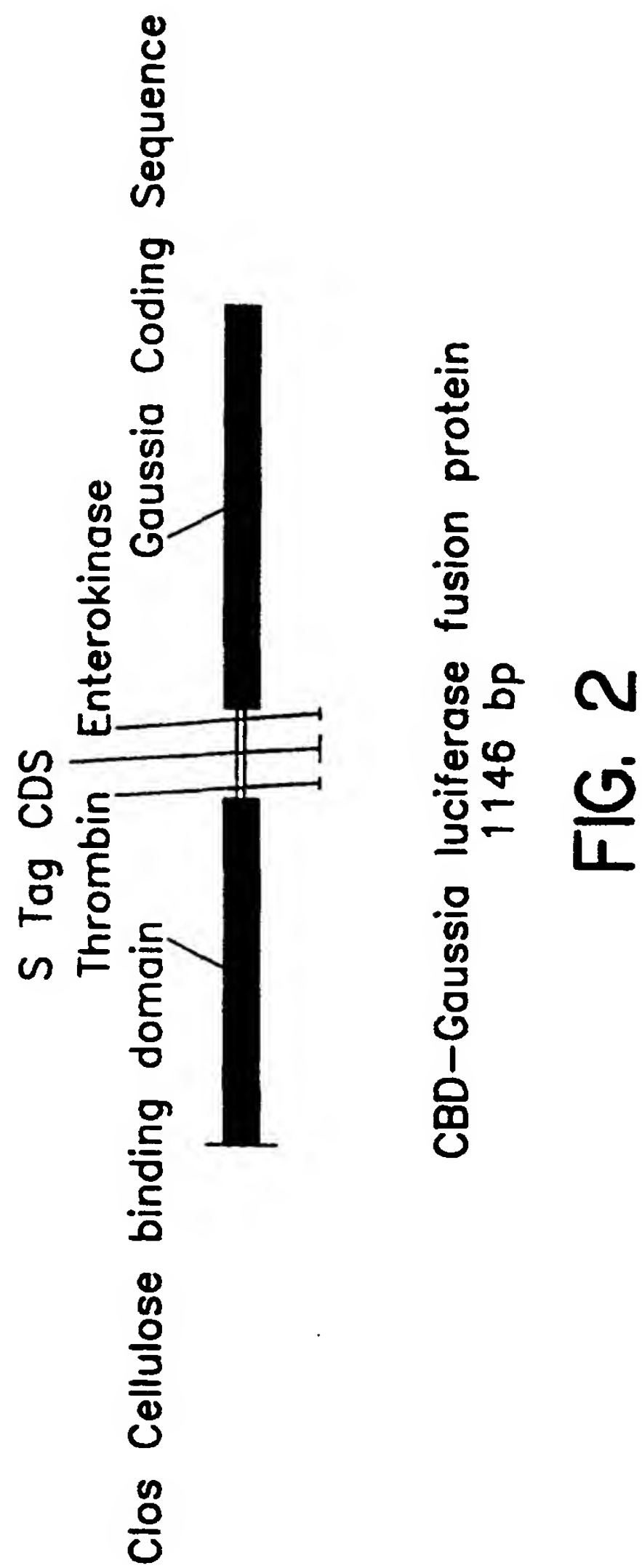


FIG. 1



R. reniformis	MDLAKLGLKEVMPTKINLEGLVGDHAFSMEGVGEGNILEG	VKIS
R. mullerei	1 MSKQILKNTCLQEVMSYKVNLEGI VNNHVFTMEGCGKNILFGNQLVQIR	
R. reniformis	VTKGAPLPFAFDIVSVAF	AYTGYPEEIS DYFLQSFPEGFTYERg
R. mullerei	51 VTKGAPLPFAFDIVSPAFAQYGNRTFTKYPNDIS--DYFIQSFPAQFMAYER	
R. reniformis	NIRYQDGGTAIVKS	
R. mullerei	99 TLRVEDGGLVEIRSDINLIEDKFVYRVEYKGSNFPDDGPMQKTILGIEP	
R. reniformis	eSMY TNVTSVIGqIIAEKL	QTGKHFTYHMRVTYKSKKPVETMPLYH
R. mullerei	149 SFEAMYMNNGVLVGEVILVYKL-NSGKYYSCHMKTLMKSKGVVKEFP SYH	
R. reniformis	FIQHRL VKTNVDTASGYVVQHETAIAAHSTIKKIEgaar	
R. mullerei	198 FIQHRL--EKT YVED-GGFVEQHETAIAQMTSIGKPLGSLHEWV	

FIG. 3

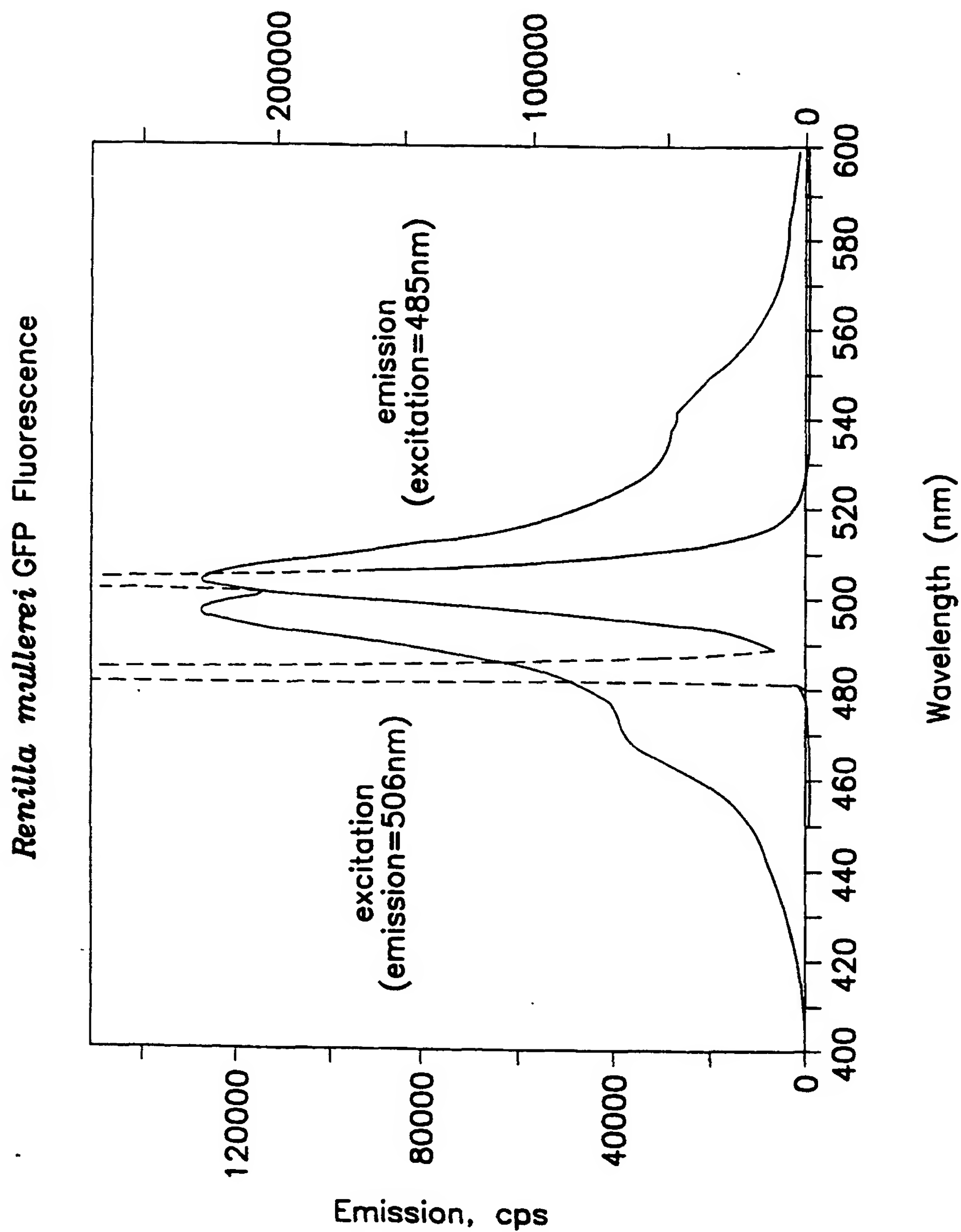


FIG. 4

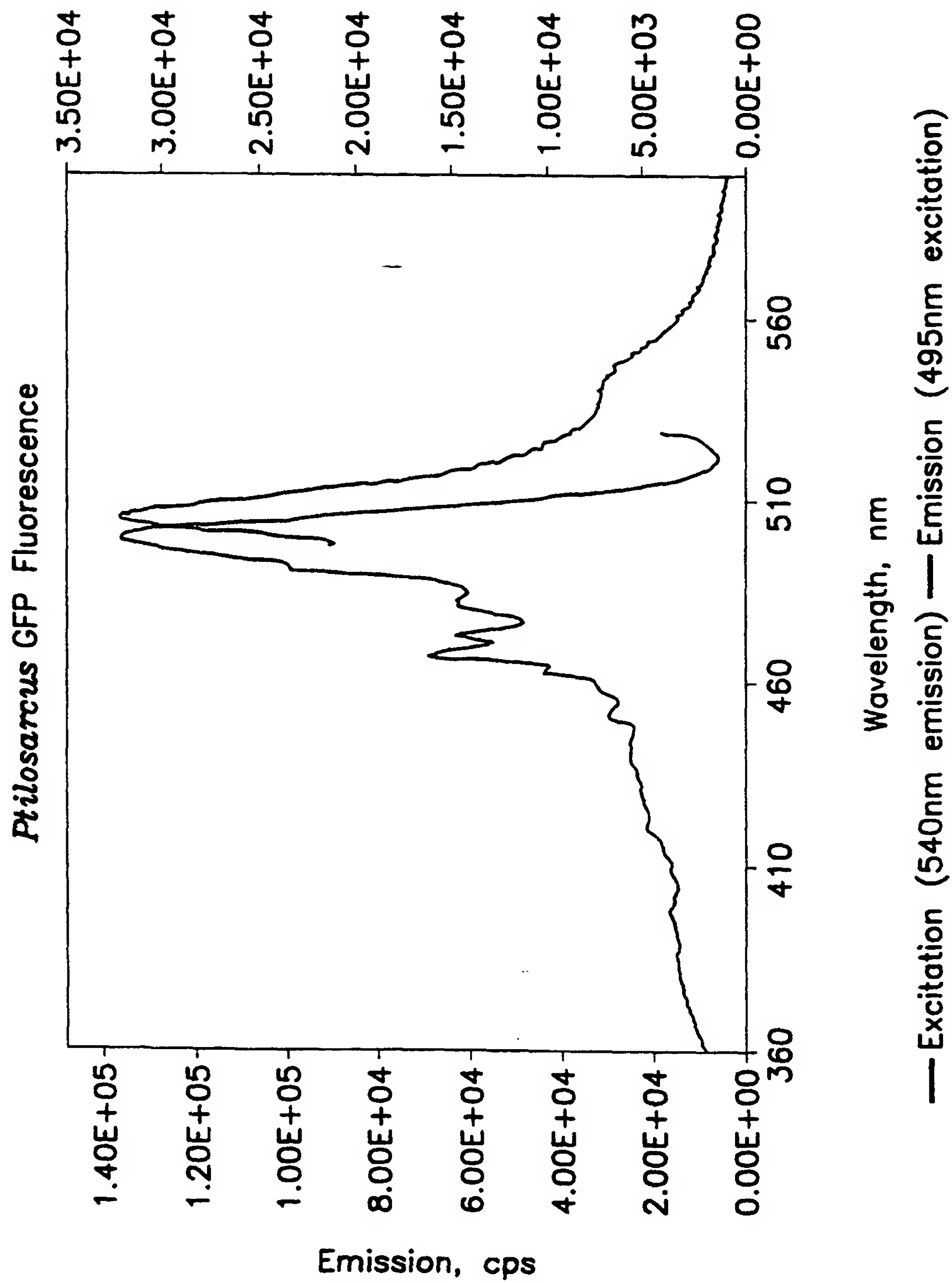


FIG. 5

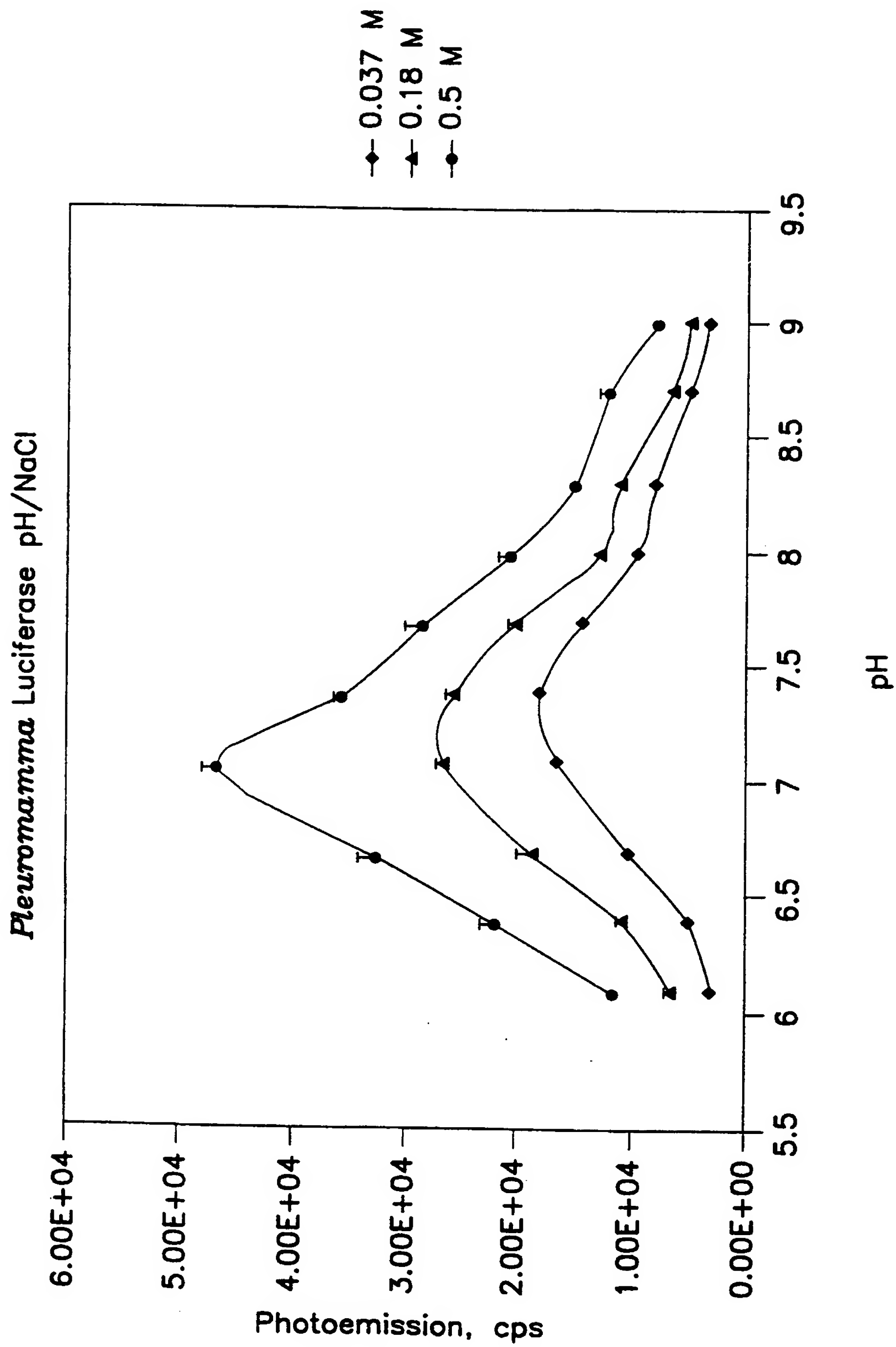
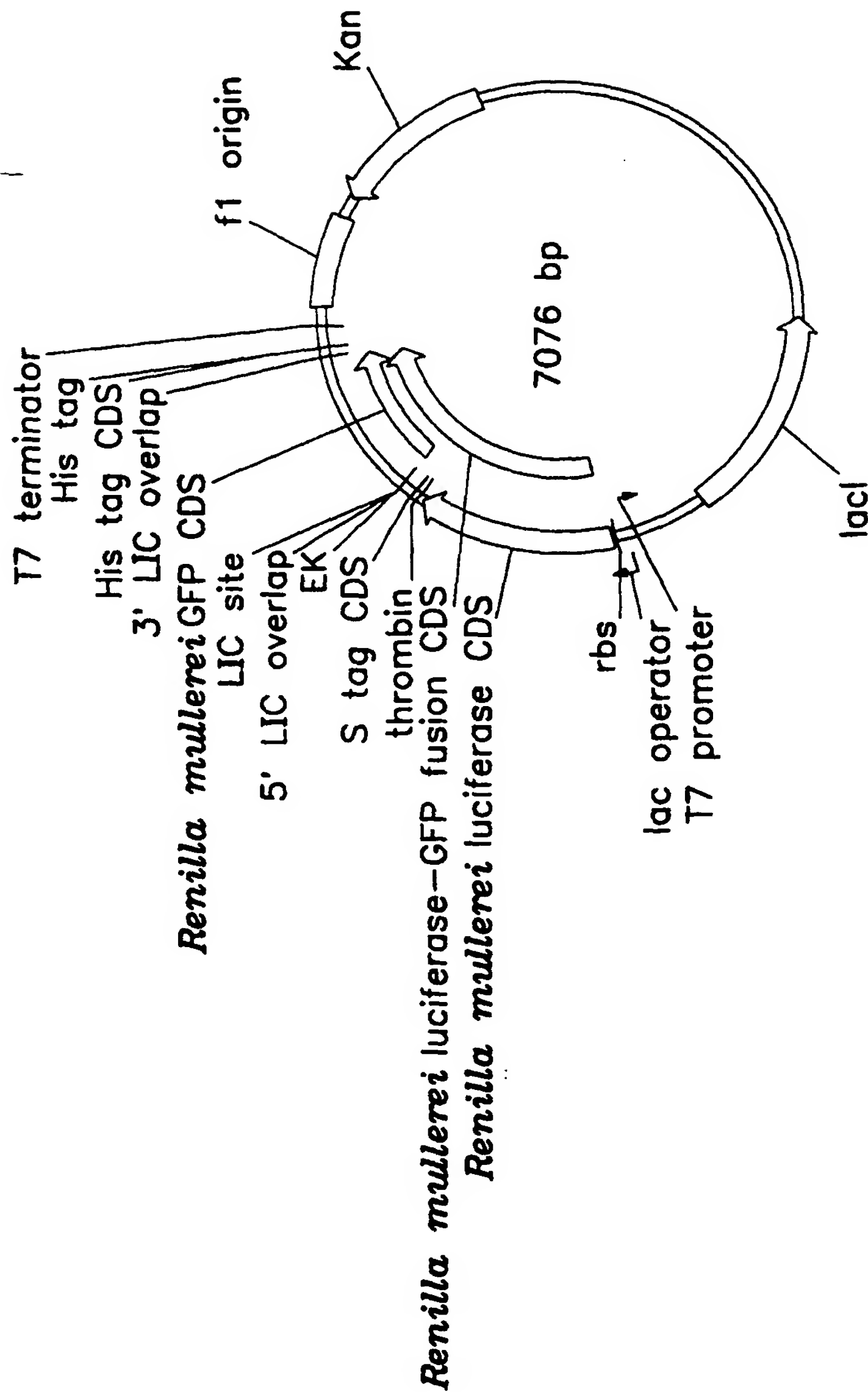


FIG. 6



pET-34 LIC *Renilla mullerei* luciferase-GFP fusion

FIG. 7

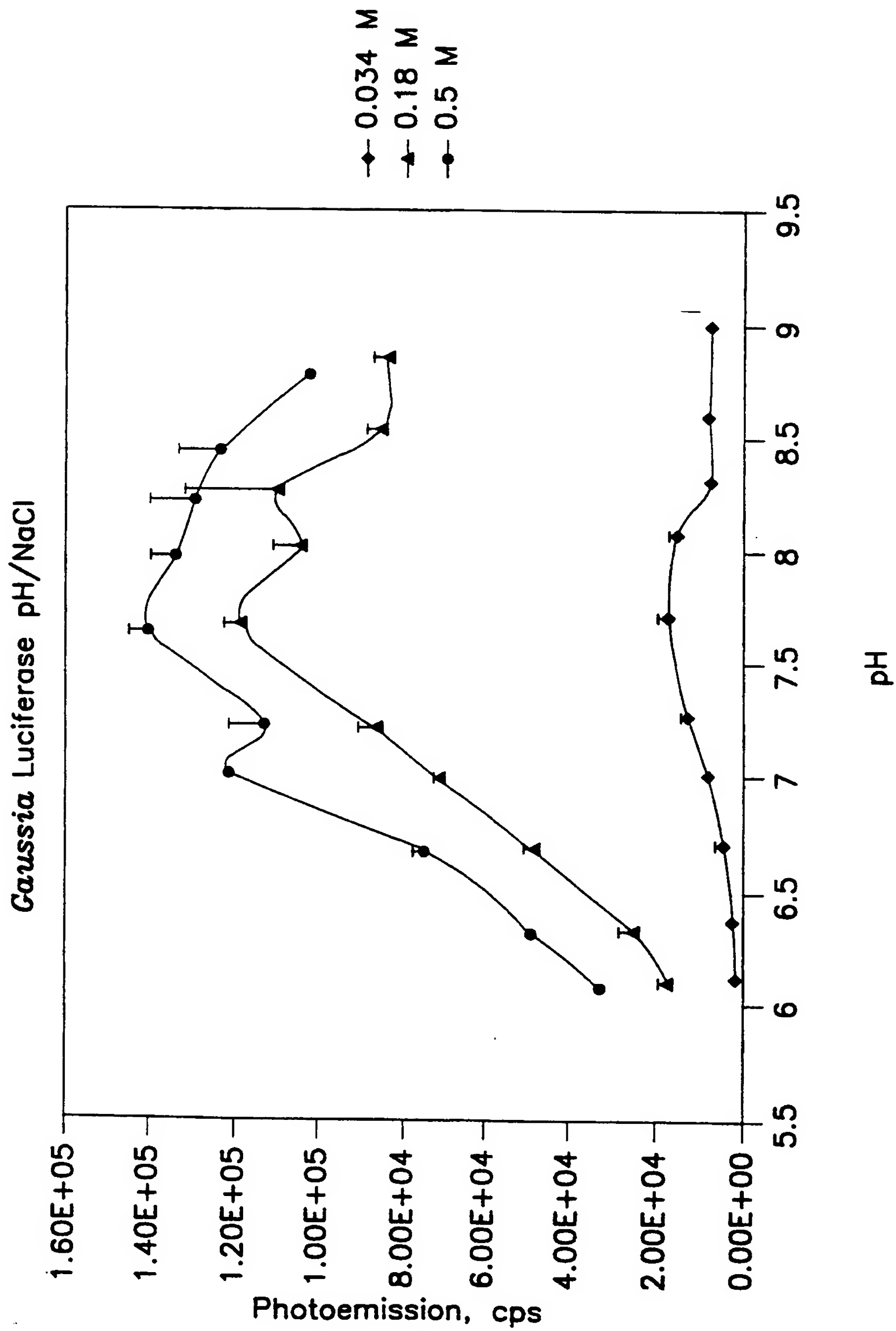


FIG. 8

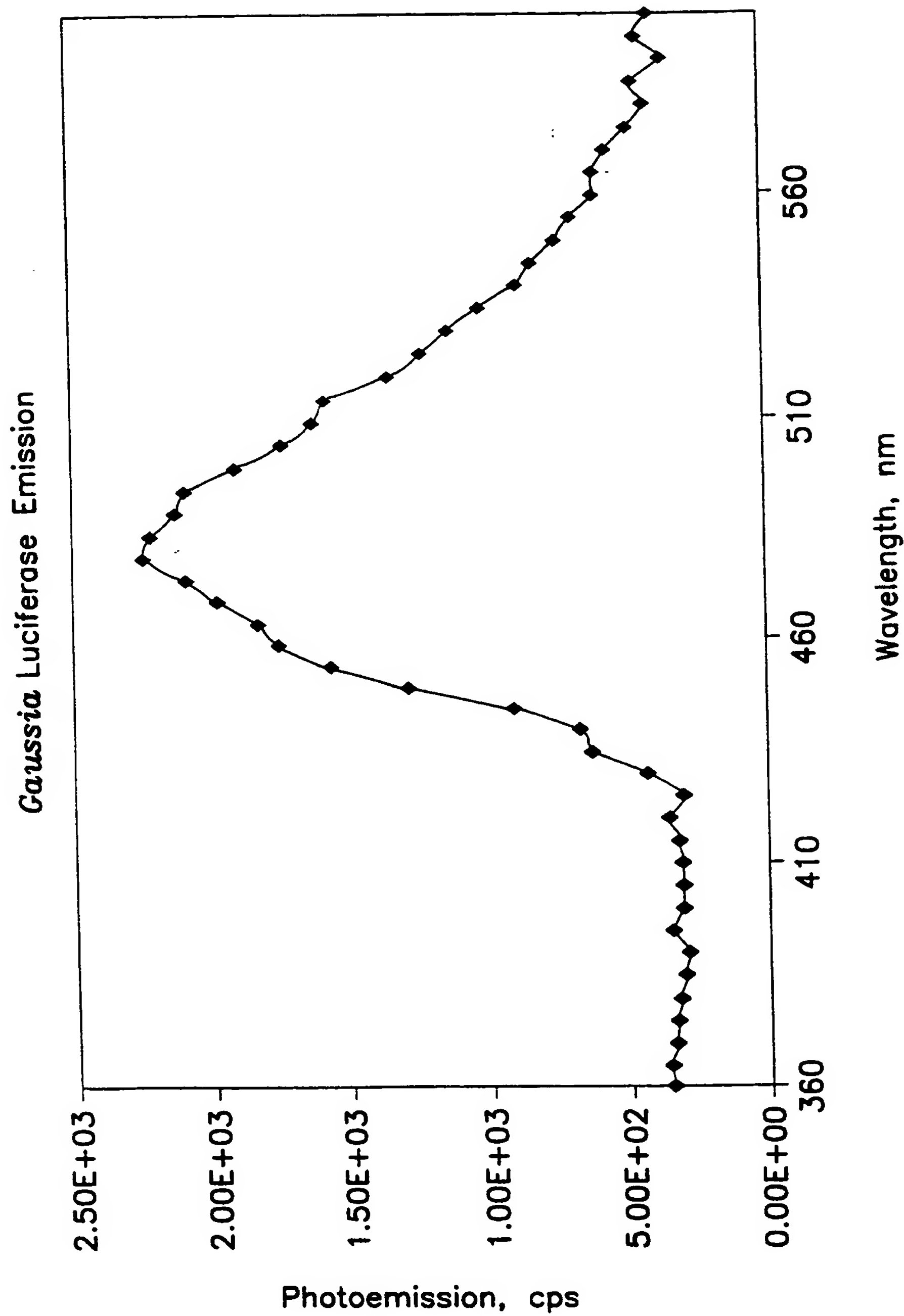


FIG. 9

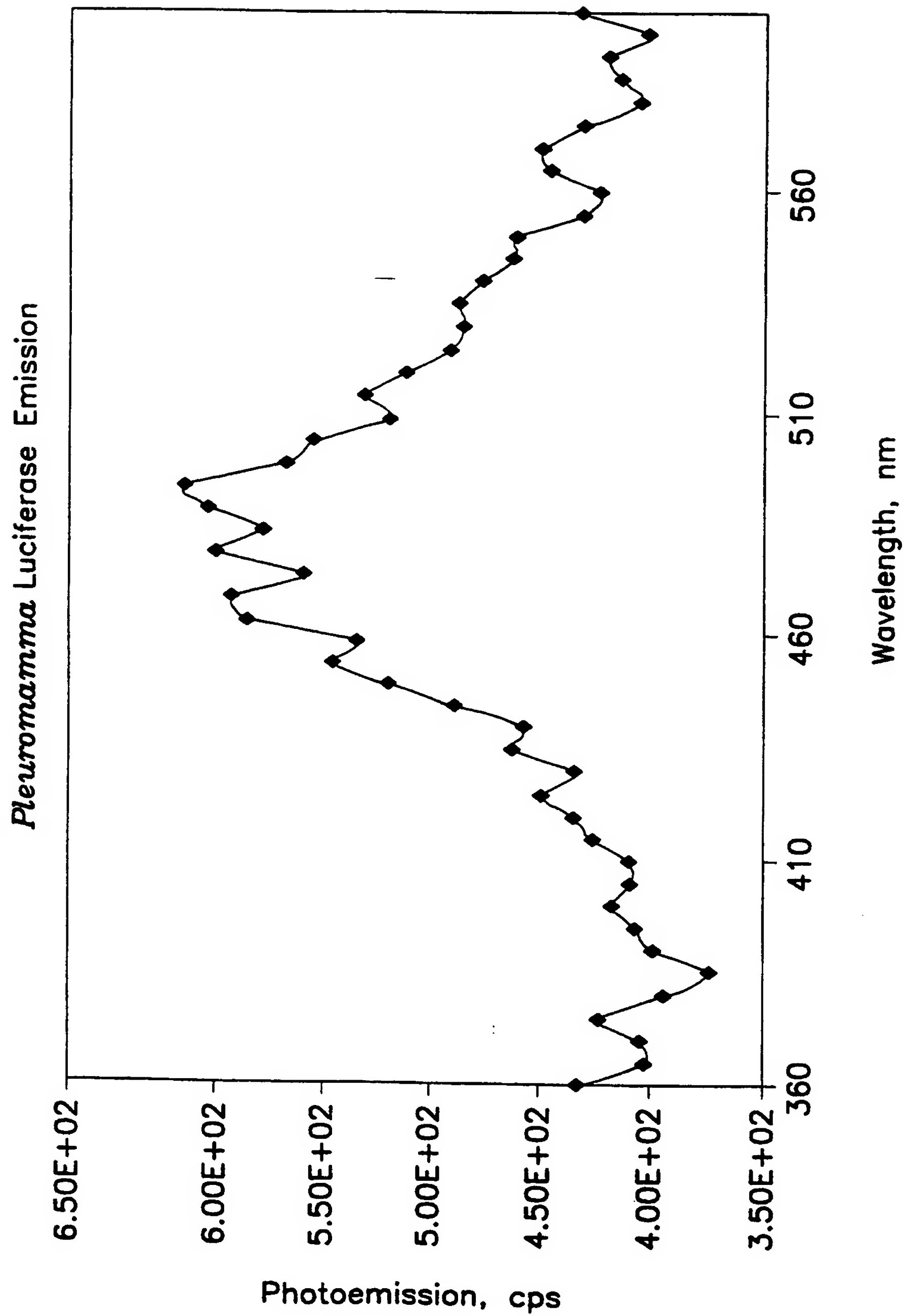


FIG. 10

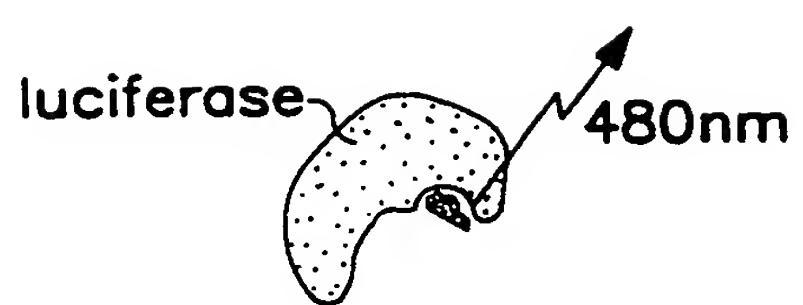


FIG. 1 IA

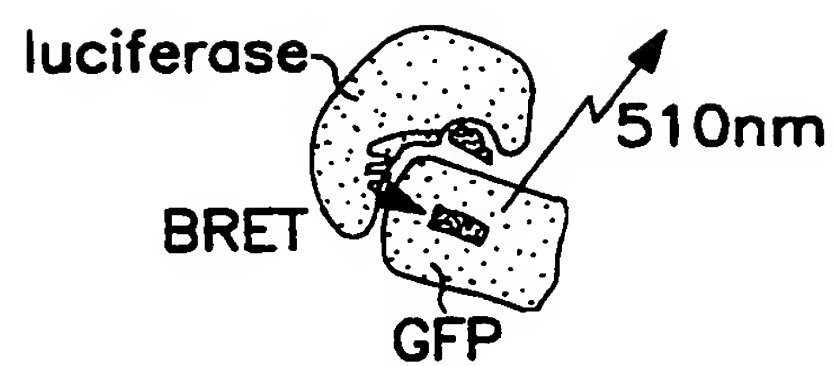


FIG. 1 IC

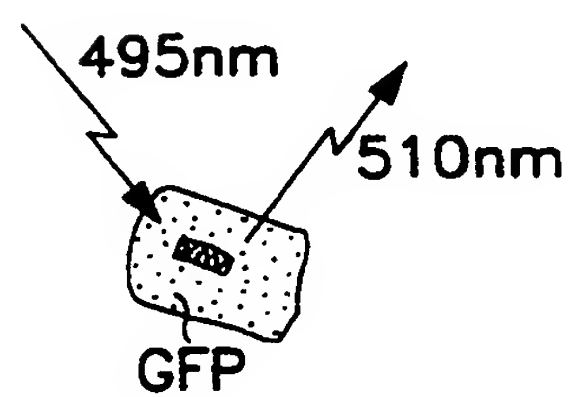


FIG. 1 IB

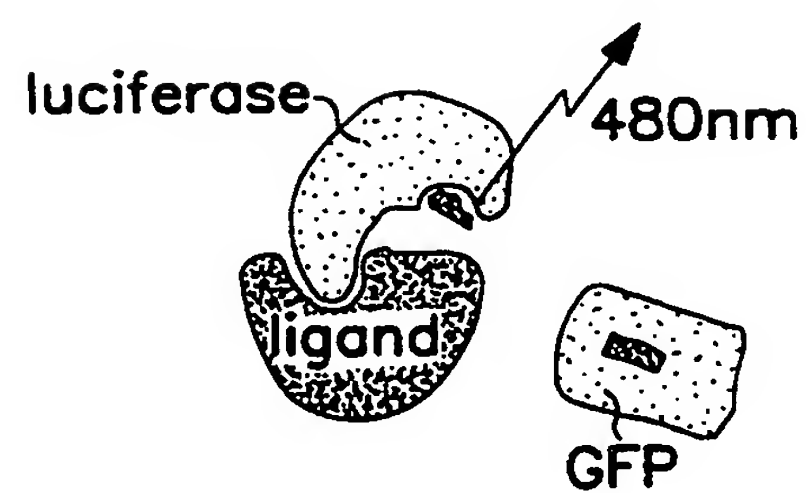


FIG. 1 ID

-1/43-

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT:

(A) NAME: PROLUME, LTD.
(B) STREET: 1085 William Pitt Way
(D) STATE: Pennsylvania
(D) COUNTRY: USA
(E) POSTAL CODE (ZIP): 15236

(i) INVENTOR/APPLICANT:

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(D) STATE: California
(D) COUNTRY: USA
(E) POSTAL CODE (ZIP): 90210

(i) INVENTOR/APPLICANT:

(A) NAME: Szent-Gyorgyi, Christopher
(B) STREET: 719 Duncan Avenue
(C) CITY: Pittsburgh
(D) STATE: Pennsylvania
(D) COUNTRY: USA
(E) POSTAL CODE (ZIP): 15237

(ii) TITLE OF INVENTION: LUCIFERASES, FLUORESCENT PROTEINS,
NUCLEIC ACIDS ENCODING THE LUCIFERASES
AND FLUORESCENT PROTEINS AND THE USE
THEREOF IN DIAGNOSTICS, HIGH THROUGHPUT
SCREENING AND NOVELTY ITEMS

(iii) NUMBER OF SEQUENCES: 32

(iv) CORRESPONDENCE ADDRESS:

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(B) STREET: 4250 Executive Square, 7th Floor
(C) CITY: La Jolla
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 92037

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE:
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ Version 1.5 and ASCII

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE: 26-MAR-1999
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 60/102,939
(B) FILING DATE: 01-OCT-1998

(vii) PRIOR APPLICATION DATA:

-2/43-

- (A) APPLICATION NUMBER: 60/089,367
 (B) FILING DATE: 15-JUNE-1998

- (vii) PRIOR APPLICATION DATA:
 (A) APPLICATION NUMBER: 60/079,624
 (B) FILING DATE: 27-MAR-1998

- (viii) ATTORNEY/AGENT INFORMATION:
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- (ix) TELECOMMUNICATION INFORMATION:
 (A) TELEPHONE: 619 450-8400
 (B) TELEFAX: 619-450-8499
 (C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1196 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (vi) ORIGINAL SOURCE:

- (ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
 (B) LOCATION: 1...942
 (D) OTHER INFORMATION: Renilla Reinformis Luciferase

- (x) PUBLICATION INFORMATION:

PATENT NO.: 5,418,155

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGC	TTA	AAG	ATG	ACT	TCG	AAA	GTT	TAT	GAT	CCA	GAA	CAA	AGG	AAA	CGG	48
Ser	Leu	Lys	Met	Thr	Ser	Lys	Val	Tyr	Asp	Pro	Glu	Gln	Arg	Lys	Arg	
1				5				10						15		
ATG	ATA	ACT	GGT	CCG	CAG	TGG	TGG	GCC	AGA	TGT	AAA	CAA	ATG	AAT	GTT	96
Met	Ile	Thr	Gly	Pro	Gln	Trp	Trp	Ala	Arg	Cys	Lys	Gln	Met	Asn	Val	
			20					25					30			
CTT	GAT	TCA	TTT	ATT	AAT	TAT	TAT	GAT	TCA	GAA	AAA	CAT	GCA	GAA	AAT	144
Leu	Asp	Ser	Phe	Ile	Asn	Tyr	Tyr	Asp	Ser	Glu	Lys	His	Ala	Glu	Asn	
		35				40						45				
GCT	GTT	ATT	TTT	TTA	CAT	GGT	AAC	GCG	GCC	TCT	TCT	TAT	TTA	TGG	CGA	192
Ala	Val	Ile	Phe	Leu	His	Gly	Asn	Ala	Ala	Ser	Ser	Tyr	Leu	Trp	Arg	
		50				55					60					
CAT	GTT	GTG	CCA	CAT	ATT	GAG	CCA	GTA	GCG	CGG	TGT	ATT	ATA	CCA	GAT	240
His	Val	Val	Pro	His	Ile	Glu	Pro	Val	Ala	Arg	Cys	Ile	Ile	Pro	Asp	
65					70					75					80	

-3/43-

CTT	ATT	GGT	ATG	GGC	AAA	TCA	GGC	AAA	TCT	GGT	AAT	GGT	TCT	TAT	AGG	288
Leu	Ile	Gly	Met	Gly	Lys	Ser	Gly	Lys	Ser	Gly	Asn	Gly	Ser	Tyr	Arg	
				85					90					95		
TTA	CTT	GAT	CAT	TAC	AAA	TAT	CTT	ACT	GCA	TGG	TTG	AAC	TTC	TTA	ATT	336
Leu	Leu	Asp	His	Tyr	Lys	Tyr	Leu	Thr	Ala	Trp	Leu	Asn	Phe	Leu	Ile	
			100					105					110			
TAC	CAA	AGA	AGA	TCA	TTT	TTT	GTC	GGC	CAT	GAT	TGG	GGT	GCT	TGT	TTG	384
Tyr	Gln	Arg	Arg	Ser	Phe	Phe	Val	Gly	His	Asp	Trp	Gly	Ala	Cys	Leu	
		115					120					125				
GCA	TTT	CAT	TAT	AGC	TAT	GAG	CAT	CAA	GAT	AAG	ATC	AAA	GCA	ATA	GTT	432
Ala	Phe	His	Tyr	Ser	Tyr	Glu	His	Gln	Asp	Lys	Ile	Lys	Ala	Ile	Val	
	130					135					140					
CAC	GCT	GAA	AGT	GTA	GTA	GAT	GTG	ATT	GAA	TCA	TGG	GAT	GAA	TGG	CCT	480
His	Ala	Glu	Ser	Val	Val	Asp	Val	Ile	Glu	Ser	Trp	Asp	Glu	Trp	Pro	
145					150					155					160	
GAT	ATT	GAA	GAA	GAT	ATT	GCG	TTG	ATC	AAA	TCT	GAA	GAA	GGA	GAA	AAA	528
Asp	Ile	Glu	Glu	Asp	Ile	Ala	Leu	Ile	Lys	Ser	Glu	Glu	Gly	Glu	Lys	
				165					170					175		
ATG	GTT	TTG	GAG	AAT	AAC	TTC	TTC	GTG	GAA	ACC	ATG	TTG	CCA	TCA	AAA	576
Met	Val	Leu	Glu	Asn	Asn	Phe	Phe	Val	Glu	Thr	Met	Leu	Pro	Ser	Lys	
			180					185					190			
ATC	ATG	AGA	AAG	TTA	GAA	CCA	GAA	GAA	TTT	GCA	GCA	TAT	CTT	GAA	CCA	624
Ile	Met	Arg	Lys	Leu	Glu	Pro	Glu	Glu	Phe	Ala	Ala	Tyr	Leu	Glu	Pro	
		195					200					205				
TTC	AAA	GAG	AAA	GGT	GAA	GTT	CGT	CGT	CCA	ACA	TTA	TCA	TGG	CCT	CGT	672
Phe	Lys	Glu	Lys	Gly	Glu	Val	Arg	Arg	Pro	Thr	Leu	Ser	Trp	Pro	Arg	
	210					215					220					
GAA	ATC	CCG	TTA	GTA	AAA	GGT	GGT	AAA	CCT	GAC	GTT	GTA	CAA	ATT	GTT	720
Glu	Ile	Pro	Leu	Val	Lys	Gly	Gly	Lys	Pro	Asp	Val	Val	Gln	Ile	Val	
225					230					235					240	
AGG	AAT	TAT	AAT	GCT	TAT	CTA	CGT	GCA	AGT	GAT	GAT	TTA	CCA	AAA	ATG	768
Arg	Asn	Tyr	Asn	Ala	Tyr	Leu	Arg	Ala	Ser	Asp	Asp	Leu	Pro	Lys	Met	
				245					250					255		
TTT	ATT	GAA	TCG	GAT	CCA	GGA	TTC	TTT	TCC	AAT	GCT	ATT	GTT	GAA	GGC	816
Phe	Ile	Glu	Ser	Asp	Pro	Gly	Phe	Phe	Ser	Asn	Ala	Ile	Val	Glu	Gly	
			260					265					270			
GCC	AAG	AAG	TTT	CCT	AAT	ACT	GAA	TTT	GTC	AAA	GTA	AAA	GGT	CTT	CAT	864
Ala	Lys	Lys	Phe	Pro	Asn	Thr	Glu	Phe	Val	Lys	Val	Lys	Gly	Leu	His	
		275					280					285				
TTT	TCG	CAA	GAA	GAT	GCA	CCT	GAT	GAA	ATG	GGA	AAA	TAT	ATC	AAA	TCG	912
Phe	Ser	Gln	Glu	Asp	Ala	Pro	Asp	Glu	Met	Gly	Lys	Tyr	Ile	Lys	Ser	
	290					295					300					
TTC	GTT	GAG	CGA	GTT	CTC	AAA	AAT	GAA	CAA	TAA	TTACTTTGGT	TTTTTATTTA				965
Phe	Val	Glu	Arg	Val	Leu	Lys	Asn	Glu	Gln							
305					310											
CATTTTTC	GGTTTA	ATAAAT	GT	CATTTT	CAAC	AATTTT	ATTT	TA	ACTGA	ATA						1025

-4/43-

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TTTCACAGGG AACATTCATA TATGTTGATT AATTTAGCTC GAACTTTACT CTGTCATATC 1085
ATTTTGGGAAT ATTACCTCTT TCAATGAAAC TTTATAAACA GTGGTTCAAT TAATTAATAT 1145
ATATTATAAT TACATTTGTT ATGTAATAAA CTCGGTTTAA TTATAAAAAA A 1196

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1822 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
- (B) LOCATION: 1...1665
- (D) OTHER INFORMATION: Cypridina hilgendorffii luciferase

(x) PUBLICATION INFORMATION:

PATENT NO.: EP 0 387 355 TORAY

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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ATG AAG CTA ATA ATT CTG TCT ATT ATA TTG GCC TAC TGT GTC ACA GTC      48
Met Lys Leu Ile Ile Leu Ser Ile Ile Leu Ala Tyr Cys Val Thr Val
  1             5             10             15

AAC TGC CAG GAT GCA TGT CCT GTA GAA GCT GAA GCA CCG TCA AGT ACA      96
Asn Cys Gln Asp Ala Cys Pro Val Glu Ala Glu Ala Pro Ser Ser Thr
             20             25             30

CCA ACA GTC CCA ACA TCT TGT GAA GCT AAA GAA GGA GAA TGT ATC GAT     144
Pro Thr Val Pro Thr Ser Cys Glu Ala Lys Glu Gly Glu Cys Ile Asp
             35             40             45

ACC AGA TGC GCA ACA TGT AAA CGA GAC ATA CTA TCA GAC GGA CTG TGT     192
Thr Arg Cys Ala Thr Cys Lys Arg Asp Ile Leu Ser Asp Gly Leu Cys
             50             55             60

GAA AAT AAA CCA GGG AAG ACA TGC TGT AGA ATG TGC CAG TAT GTA ATT     240
Glu Asn Lys Pro Gly Lys Thr Cys Cys Arg Met Cys Gln Tyr Val Ile
             65             70             75             80

GAA TCC AGA GTA GAA GCT GCT GGA TAT TTT AGA ACG TTT TAC GCC AAA     288
Glu Ser Arg Val Glu Ala Ala Gly Tyr Phe Arg Thr Phe Tyr Ala Lys
             85             90             95

AGA TTT AAT TTT CAG GAA CCT GGT AAA TAT GTG CTG GCT CGA GGA ACC     336
Arg Phe Asn Phe Gln Glu Pro Gly Lys Tyr Val Leu Ala Arg Gly Thr
             100            105            110

AAG GGT GGC GAC TGG TCT GTA ACC CTC ACC ATG GAG AAT CTA GAT GGA     384
Lys Gly Gly Asp Trp Ser Val Thr Leu Thr Met Glu Asn Leu Asp Gly
             115            120            125

CAG AAG GGA GCT GTA CTG ACT AAG ACA ACA CTG GAG GTA GTA GGA GAC     432
Gln Lys Gly Ala Val Leu Thr Lys Thr Thr Leu Glu Val Val Gly Asp
             130            135            140

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-5/43-

GTA Val 145	ATA Ile	GAC Asp	ATT Ile	ACT Thr	CAA Gln 150	GCT Ala	ACT Thr	GCA Ala	GAT Asp	CCT Pro 155	ATC Ile	ACA Thr	GTT Val	AAC Asn	GGA Gly 160	480
GGA Gly	GCT Ala	GAC Asp	CCA Pro	GTT Val 165	ATC Ile	GCT Ala	AAC Asn	CCG Pro	TTC Phe 170	ACA Thr	ATT Ile	GGT Gly	GAG Glu	GTG Val 175	ACC Thr	528
ATT Ile	GCT Ala	GTT Val	GTC Val 180	GAA Glu	ATA Ile	CCC Pro	GGC Gly	TTC Phe 185	AAT Asn	ATT Ile	ACA Thr	GTC Val	ATC Ile 190	GAA Glu	TTC Phe	576
TTT Phe	AAA Lys	CTA Leu 195	ATC Ile	GTG Val	ATA Ile	GAT Asp	ATT Ile 200	CTG Leu	GGA Gly	GGA Gly	AGA Arg	TCT Ser 205	GTG Val	AGA Arg	ATT Ile	624
GCT Ala 210	CCA Pro	GAC Asp	ACA Thr	GCA Ala	AAC Asn	AAA Lys 215	GGA Gly	CTG Leu	ATA Ile	TCT Ser 220	GGT Gly	ATC Ile	TGT Cys	GGT Gly	AAT Asn	672
CTG Leu 225	GAG Glu	ATG Met	AAT Asn	GAC Asp	GCT Ala 230	GAT Asp	GAC Asp	TTT Phe	ACT Thr	ACA Thr 235	GAC Asp	GCA Ala	GAT Asp	CAG Gln	CTG Leu 240	720
GCG Ala	ATC Ile	CAA Gln	CCC Pro	AAC Asn 245	ATA Ile	AAC Asn	AAA Lys	GAG Glu 250	TTC Phe	GAC Asp	GGC Gly	TGC Cys	CCA Pro	TTC Phe 255	TAC Tyr	768
GGG Gly	AAT Asn	CCT Pro	TCT Ser 260	GAT Asp	ATC Ile	GAA Glu	TAC Tyr	TGC Cys 265	AAA Lys	GGT Gly	CTC Leu	ATG Met 270	GAG Glu	CCA Pro	TAC Tyr	816
AGA Arg	GCT Ala	GTA Val 275	TGT Cys	CGT Arg	AAC Asn	AAT Asn	ATC Ile 280	AAC Asn	TTC Phe	TAC Tyr	TAT Tyr 285	TAC Tyr	ACT Thr	CTG Leu	TCC Ser	864
TGC Cys 290	GCC Ala	TTC Phe	GCT Ala	TAC Tyr	TGT Cys	ATG Met 295	GGA Gly	GGA Gly	GAA Glu	GAA Glu	AGA Arg 300	GCT Ala	AAA Lys	CAC His	GTC Val	912
CTT Leu 305	TTC Phe	GAC Asp	TAT Tyr	GTT Val	GAG Glu 310	ACA Thr	TGC Cys	GCT Ala	GCA Ala	CCG Pro 315	GAA Glu	ACG Thr	AGA Arg	GGA Gly	ACG Thr 320	960
TGT Cys	GTT Val	TTA Leu	TCA Ser	GGA Gly 325	CAT His	ACT Thr	TTC Phe	TAT Tyr	GAC Asp 330	ACA Thr	TTC Phe	GAC Asp	AAA Lys	GCC Ala 335	AGA Arg	1008
TAT Tyr	CAA Gln	TTC Phe	CAG Gln 340	GGC Gly	CCA Pro	TGC Cys	AAA Lys	GAG Glu 345	CTT Leu	CTG Leu	ATG Met	GCC Ala	GCA Ala	GAC Asp	TGT Cys	1056
TAC Tyr	TGG Trp	AAC Asn 355	ACA Thr	TGG Trp	GAT Asp	GTA Val	AAG Lys 360	GTT Val	TCA Ser	CAT His	AGA Arg 365	GAT Asp	GTT Val	GAG Glu	TCA Ser	1104
TAC Tyr 370	ACT Thr	GAG Glu	GTA Val	GAG Glu	AAA Lys	GTA Val 375	ACA Thr	ATC Ile	AGG Arg	AAA Lys	CAG Gln 380	TCA Ser	ACT Thr	GTA Val	GTA Val	1152
GAT 1200	TTG	ATT	GTG	GAT	GGC	AAG	CAG	GTC	AAG	GTT	GGA	GGA	GTG	GAT	GTA	

-6/43-

Asp 385	Leu	Ile	Val	Asp 390	Gly	Lys	Gln	Val	Lys 395	Val	Gly	Gly	Val	Asp 400	Val		
TCT	ATC	CCG	TAC	AGT	TCT	GAG	AAC	ACA	TCC	ATA	TAC	TGG	CAG	GAT	GGA	1248	
Ser	Ile	Pro	Tyr	Ser 405	Ser	Glu	Asn	Thr 410	Ser	Ile	Tyr	Trp	Gln	Asp 415	Gly		
GAC	ATC	CTG	ACG	ACG	GCC	ATC	CTA	CCT	GAA	GCT	CTT	GTC	GTT	AAG	TTC	1296	
Asp	Ile	Leu	Thr 420	Thr	Ala	Ile	Leu 425	Pro	Glu	Ala	Leu	Val	Val 430	Lys	Phe		
AAC	TTT	AAG	CAG	CTC	CTT	GTA	GTT	CAT	ATC	AGA	GAT	CCA	TTC	GAT	GGA	1344	
Asn	Phe	Lys 435	Gln	Leu	Leu	Val 440	Val	His	Ile	Arg	Asp 445	Pro	Phe	Asp	Gly		
AAG	ACA	TGC	GGC	ATA	TGT	GGT	AAC	TAT	AAT	CAA	GAT	TCA	ACT	GAT	GAT	1392	
Lys 450	Thr	Cys	Gly	Ile	Cys	Gly 455	Asn	Tyr	Asn	Gln 460	Asp	Ser	Thr	Asp	Asp		
TTC	TTT	GAC	GCA	GAA	GGA	GCA	TGC	GCT	CTG	ACC	CCC	AAT	CCC	CCA	GGA	1440	
Phe 465	Phe	Asp	Ala	Glu	Gly 470	Ala	Cys	Ala	Leu	Thr 475	Pro	Asn	Pro	Pro	Gly 480		
TGT	ACA	GAG	GAG	CAG	AAA	CCA	GAA	GCT	GAG	CGA	CTC	TGC	AAT	AGT	CTA	1488	
Cys	Thr	Glu	Glu	Gln 485	Lys	Pro	Glu	Ala 490	Glu	Arg	Leu	Cys	Asn	Ser	Leu		
TTT	GAT	AGT	TCT	ATC	GAC	GAG	AAA	TGT	AAT	GTC	TGC	TAC	AAG	CCT	GAC	1536	
Phe	Asp	Ser 500	Ser	Ile	Asp	Glu	Lys 505	Cys	Asn	Val	Cys	Tyr 510	Lys	Pro	Asp		
CGT	ATT	GCA	CGA	TGT	ATG	TAC	GAG	TAT	TGC	CTG	AGG	GGA	CAG	CAA	GGA	1584	
Arg	Ile	Ala 515	Arg	Cys	Met	Tyr	Glu 520	Tyr	Cys	Leu	Arg	Gly 525	Gln	Gln	Gly		
TTC	TGT	GAC	CAT	GCT	TGG	GAG	TTC	AAA	AAA	GAA	TGC	TAC	ATA	AAG	CAT	1632	
Phe 530	Cys	Asp	His	Ala	Trp	Glu 535	Phe	Lys	Lys	Glu 540	Cys	Tyr	Ile	Lys	His		
GGA	GAC	ACT	CTA	GAA	GTA	CCA	CCT	GAA	TGC	CAA	TAA	ATGAACAAAG				1678	
Gly 545	Asp	Thr	Leu	Glu 550	Val	Pro	Pro	Glu	Cys	Gln 555							
ATACAGAAGC	TAAGACTACT	ACAGCAGAAG	ATAAAAGAGA	AGCTGTAGTT	CTTCAAAAAC											1738	
AGTATATTTT	GATGTACTCA	TTGTTTACTT	ACATAAAAAT	AAATTGTTAT	TATCATAACG											1798	
TAAAGAAAAA	AAAAAAAAAA	AAAA														1822	

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1644 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
- (B) LOCATION: 1...1644

-7/43-

(D) OTHER INFORMATION: *Luciola Cruciata* Luciferase (Firefly)

(x) PUBLICATION INFORMATION:

PATENT NO.: 4,968,613

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATG	GAA	AAC	ATG	GAA	AAC	GAT	GAA	AAT	ATT	GTA	GTT	GGA	CCT	AAA	CCG	48
Met	Glu	Asn	Met	Glu	Asn	Asp	Glu	Asn	Ile	Val	Val	Gly	Pro	Lys	Pro	
1				5					10					15		
TTT	TAC	CCT	ATC	GAA	GAG	GGA	TCT	GCT	GGA	ACA	CAA	TTA	CGC	AAA	TAC	96
Phe	Tyr	Pro	Ile	Glu	Glu	Gly	Ser	Ala	Gly	Thr	Gln	Leu	Arg	Lys	Tyr	
			20					25					30			
ATG	GAG	CGA	TAT	GCA	AAA	CTT	GGC	GCA	ATT	GCT	TTT	ACA	AAT	GCA	GTT	144
Met	Glu	Arg	Tyr	Ala	Lys	Leu	Gly	Ala	Ile	Ala	Phe	Thr	Asn	Ala	Val	
		35					40					45				
ACT	GGT	GTT	GAT	TAT	TCT	TAC	GCC	GAA	TAC	TTG	GAG	AAA	TCA	TGT	TGT	192
Thr	Gly	Val	Asp	Tyr	Ser	Tyr	Ala	Glu	Tyr	Leu	Glu	Lys	Ser	Cys	Cys	
	50					55					60					
CTA	GGA	AAA	GCT	TTG	CAA	AAT	TAT	GGT	TTG	GTT	GTT	GAT	GGC	AGA	ATT	240
Leu	Gly	Lys	Ala	Leu	Gln	Asn	Tyr	Gly	Leu	Val	Val	Asp	Gly	Arg	Ile	
65					70				75						80	
GCG	TTA	TGC	AGT	GAA	AAC	TGT	GAA	GAA	TTT	TTT	ATT	CCT	GTA	ATA	GCC	288
Ala	Leu	Cys	Ser	Glu	Asn	Cys	Glu	Glu	Phe	Phe	Ile	Pro	Val	Ile	Ala	
				85					90					95		
GGA	CTG	TTT	ATA	GGT	GTA	GGT	GTT	GCA	CCC	ACT	AAT	GAG	ATT	TAC	ACT	336
Gly	Leu	Phe	Ile	Gly	Val	Gly	Val	Ala	Pro	Thr	Asn	Glu	Ile	Tyr	Thr	
			100					105					110			
TTA	CGT	GAA	CTG	GTT	CAC	AGT	TTA	GGT	ATC	TCT	AAA	CCA	ACA	ATT	GTA	384
Leu	Arg	Glu	Leu	Val	His	Ser	Leu	Gly	Ile	Ser	Lys	Pro	Thr	Ile	Val	
		115					120					125				
TTT	AGT	TCT	AAA	AAA	GGC	TTA	GAT	AAA	GTT	ATA	ACA	GTA	CAG	AAA	ACA	432
Phe	Ser	Ser	Lys	Lys	Gly	Leu	Asp	Lys	Val	Ile	Thr	Val	Gln	Lys	Thr	
	130					135					140					
GTA	ACT	ACT	ATT	AAA	ACC	ATT	GTT	ATA	CTA	GAT	AGC	AAA	GTT	GAT	TAT	480
Val	Thr	Thr	Ile	Lys	Thr	Ile	Val	Ile	Leu	Asp	Ser	Lys	Val	Asp	Tyr	
145					150					155					160	
CGA	GGA	TAT	CAA	TGT	CTG	GAC	ACC	TTT	ATA	AAA	AGA	AAC	ACT	CCA	CCA	528
Arg	Gly	Tyr	Gln	Cys	Leu	Asp	Thr	Phe	Ile	Lys	Arg	Asn	Thr	Pro	Pro	
				165					170					175		
GGT	TTT	CAA	GCA	TCC	AGT	TTC	AAA	ACT	GTG	GAA	GTT	GAC	CGT	AAA	GAA	576
Gly	Phe	Gln	Ala	Ser	Ser	Phe	Lys	Thr	Val	Glu	Val	Asp	Arg	Lys	Glu	
			180					185					190			
CAA	GTT	GCT	CTT	ATA	ATG	AAC	TCT	TCG	GGT	TCT	ACC	GGT	TTG	CCA	AAA	624
Gln	Val	Ala	Leu	Ile	Met	Asn	Ser	Ser	Gly	Ser	Thr	Gly	Leu	Pro	Lys	
		195					200					205				
GGC	GTA	CAA	CTT	ACT	CAC	GAA	AAT	ACA	GTC	ACT	AGA	TTT	TCT	CAT	GCT	672

-8/43-

Gly	Val	Gln	Leu	Thr	His	Glu	Asn	Thr	Val	Thr	Arg	Phe	Ser	His	Ala	
210						215					220					
AGA	GAT	CCG	ATT	TAT	GGT	AAC	CAA	GTT	TCA	CCA	GGC	ACC	GCT	GTT	TTA	720
Arg	Asp	Pro	Ile	Tyr	Gly	Asn	Gln	Val	Ser	Pro	Gly	Thr	Ala	Val	Leu	
225					230				235						240	
ACT	GTC	GTT	CCA	TTC	CAT	CAT	GGT	TTT	GGT	ATG	TTC	ACT	ACT	CTA	GGG	768
Thr	Val	Val	Pro	Phe	His	His	Gly	Phe	Gly	Met	Phe	Thr	Thr	Leu	Gly	
				245					250					255		
TAT	TTA	ATT	TGT	GGT	TTT	CGT	GTT	GTA	ATG	TTA	ACA	AAA	TTC	GAT	GAA	816
Tyr	Leu	Ile	Cys	Gly	Phe	Arg	Val	Val	Met	Leu	Thr	Lys	Phe	Asp	Glu	
			260					265					270			
GAA	ACA	TTT	TTA	AAA	ACT	CTA	CAA	GAT	TAT	AAA	TGT	ACA	AGT	GTT	ATT	864
Glu	Thr	Phe	Leu	Lys	Thr	Leu	Gln	Asp	Tyr	Lys	Cys	Thr	Ser	Val	Ile	
		275					280					285				
CTT	GTA	CCG	ACC	TTG	TTT	GCA	ATT	CTC	AAC	AAA	AGT	GAA	TTA	CTC	AAT	912
Leu	Val	Pro	Thr	Leu	Phe	Ala	Ile	Leu	Asn	Lys	Ser	Glu	Leu	Leu	Asn	
	290					295					300					
AAA	TAC	GAT	TTG	TCA	AAT	TTA	GTT	GAG	ATT	GCA	TCT	GGC	GGA	GCA	CCT	960
Lys	Tyr	Asp	Leu	Ser	Asn	Leu	Val	Glu	Ile	Ala	Ser	Gly	Gly	Ala	Pro	
305					310					315					320	
TTA	TCA	AAA	GAA	GTT	GGT	GAA	GCT	GTT	GCT	AGA	CGC	TTT	AAT	CTT	CCC	1008
Leu	Ser	Lys	Glu	Val	Gly	Glu	Ala	Val	Ala	Arg	Arg	Phe	Asn	Leu	Pro	
				325					330					335		
GGT	GTT	CGT	CAA	GGT	TAT	GGT	TTA	ACA	GAA	ACA	ACA	TCT	GCC	ATT	ATT	1056
Gly	Val	Arg	Gln	Gly	Tyr	Gly	Leu	Thr	Glu	Thr	Thr	Ser	Ala	Ile	Ile	
			340					345					350			
ATT	ACA	CCA	GAA	GGA	GAC	GAT	AAA	CCA	GGA	GCT	TCT	GGA	AAA	GTC	GTG	1104
Ile	Thr	Pro	Glu	Gly	Asp	Asp	Lys	Pro	Gly	Ala	Ser	Gly	Lys	Val	Val	
		355					360					365				
CCG	TTG	TTT	AAA	GCA	AAA	GTT	ATT	GAT	CTT	GAT	ACC	AAA	AAA	TCT	TTA	1152
Pro	Leu	Phe	Lys	Ala	Lys	Val	Ile	Asp	Leu	Asp	Thr	Lys	Lys	Ser	Leu	
	370					375					380					
GGT	CCT	AAC	AGA	CGT	GGA	GAA	GTT	TGT	GTT	AAA	GGA	CCT	ATG	CTT	ATG	1200
Gly	Pro	Asn	Arg	Arg	Gly	Glu	Val	Cys	Val	Lys	Gly	Pro	Met	Leu	Met	
385					390					395					400	
AAA	GGT	TAT	GTA	AAT	AAT	CCA	GAA	GCA	ACA	AAA	GAA	CTT	ATT	GAC	GAA	1248
Lys	Gly	Tyr	Val	Asn	Asn	Pro	Glu	Ala	Thr	Lys	Glu	Leu	Ile	Asp	Glu	
				405					410					415		
GAA	GGT	TGG	CTG	CAC	ACC	GGA	GAT	ATT	GGA	TAT	TAT	GAT	GAA	GAA	AAA	1296
Glu	Gly	Trp	Leu	His	Thr	Gly	Asp	Ile	Gly	Tyr	Tyr	Asp	Glu	Glu	Lys	
			420					425					430			
CAT	TTC	TTT	ATT	GTC	GAT	CGT	TTG	AAG	TCT	TTA	ATC	AAA	TAC	AAA	GGA	1344
His	Phe	Phe	Ile	Val	Asp	Arg	Leu	Lys	Ser	Leu	Ile	Lys	Tyr	Lys	Gly	
		435					440					445				
TAC	CAA	GTA	CCA	CCT	GCC	GAA	TTA	GAA	TCC	GTT	CTT	TTG	CAA	CAT	CCA	1392
Tyr	Gln	Val	Pro	Pro	Ala	Glu	Leu	Glu	Ser	Val	Leu	Leu	Gln	His	Pro	

-9/43-

450	455	460	
TCT ATC TTT GAT GCT GGT GTT GCC GGC GTT CCT GAT CCT GTA GCT GGC			1440
Ser Ile Phe Asp Ala Gly Val Ala Gly Val Pro Asp Pro Val Ala Gly			
465	470	475	480
GAG CTT CCA GGA GCC GTT GTT GTA CTG GAA AGC GGA AAA AAT ATG ACC			1488
Glu Leu Pro GLy Ala Val Val Val Leu Glu Ser Gly Lys Asn Met Thr			
	485	490	495
GAA AAA GAA GTA ATG GAT TAT GTT GCA AGT CAA GTT TCA AAT GCA AAA			1536
Glu Lys Glu Val Met Asp Tyr Val Als Ser Gln Val Ser Asn Ala Lys			
	500	505	510
CGT TTA CGT GGT GGT GTT CGT TTT GTG GAT GAA GTA CCT AAA GGT CTT			1584
Arg Leu Arg Gly Gly Val Arg Phe Val Asp Glu Val Pro Lys Gly Leu			
	515	520	525
ACT GGA AAA ATT GAC GGC AGA GCA ATT AGA GAA ATC CTT AAG AAA CCA			1632
Thr Gly Lys Ile Asp Gly Arg Ala Ile Arg Glu Ile Leu Lys Lys Pro			
	530	535	540
GTT GCT AAG ATG			1644
Val Ala Lys Met			
545			

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1820 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
- (B) LOCATION: 1...1664
- (D) OTHER INFORMATION: Vargula (cypridina) luciferase

(x) PUBLICATION INFORMATION:

JP 3-30678 Osaka (Tsuji)

- (A) AUTHORS: Thompson *et al.*
- (C) JOURNAL: Proc. Natl. Acad. Sci. U.S.A.
- (D) VOLUME: 86
- (F) PAGES: 1326-1332
- (G) DATE: (1989)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATG AAG ATA ATA ATT CTG TCT GTT ATA TTG GCC TAC TGT GTC ACC GAC	48
Met Lys Ile Ile Ile Leu Ser Val Ile Leu Ala Tyr Cys Val Thr Asp	
1 5 10 15	
AAC TGT CAA GAT GCA TGT CCT GTA GAA GCG GAA CCG CCA TCA AGT ACA	96
Asn Cys Gln Asp Ala Cys Pro Val Glu Ala Glu Pro Pro Ser Ser Thr	
20 25 30	
CCA ACA GTT CCA ACT TCT TGT GAA GCT AAA GAA GGA GAA TGT ATA GAT	144
Pro Thr Val Pro Thr Ser Cys Glu Ala Lys Glu Gly Glu Cys Ile Asp	

-10/43-

35	40	45	
ACC AGA TGC GCA ACA TGT AAA CGA GAT ATA CTA TCA GAT GGA CTG TGT Thr Arg Cys Ala Thr Cys Lys Arg Asp Ile Leu Ser Asp Gly Leu Cys 50 55 60			192
GAA AAT AAA CCA GGG AAG ACA TGC TGT AGA ATG TGC CAG TAT GTG ATT Glu Asn Lys Pro Gly Lys Thr Cys Cys Arg Met Cys Gln Tyr Val Ile 65 70 75 80			240
GAA TGC AGA GTA GAA GCA GCT GGT TAT TTT AGA ACG TTT TAC GGC AAA Glu Cys Arg Val Glu Ala Ala Gly Tyr Phe Arg Thr Phe Tyr Gly Lys 85 90 95			288
AGA TTT AAT TTT CAG GAA CCT GGT AAA TAT GTG CTG GCT AGG GGA ACC Arg Phe Asn Phe Gln Glu Pro Gly Lys Tyr Val Leu Ala Arg Gly Thr 100 105 110			336
AAG GGT GGC GAT TGG TCT GTA ACC CTC ACC ATG GAG AAT CTA GAT GGA Lys Gly Gly Asp Trp Ser Val Thr Leu Thr Met Glu Asn Leu Asp Gly 115 120 125			384
CAG AAG GGA GCT GTG CTG ACT AAG ACA ACA CTG GAG GTT GCA GGA GAC Gln Lys Gly Ala Val Leu Thr Lys Thr Thr Leu Glu Val Ala Gly Asp 130 135 140			432
GTA ATA GAC ATT ACT CAA GCT ACT GCA GAT CCT ATC ACA GTT AAC GGA Val Ile Asp Ile Thr Gln Ala Thr Ala Asp Pro Ile Thr Val Asn Gly 145 150 155 160			480
GGA GCT GAC CCA GTT ATC GCT AAC CCG TTC ACA ATT GGT GAG GTG ACC Gly Ala Asp Pro Val Ile Ala Asn Pro Phe Thr Ile Gly Glu Val Thr 165 170 175			528
ATT GCT GTT GTT GAA ATA CCG GGC TTC AAT ATC ACA GTC ATC GAA TTC Ile Ala Val Val Glu Ile Pro Gly Phe Asn Ile Thr Val Ile Glu Phe 180 185 190			576
TTT AAA CTA ATC GTG ATT GAT ATT CTG GGA GGA AGA TCT GTC AGA ATT Phe Lys Leu Ile Val Ile Asp Ile Leu Gly Gly Arg Ser Val Arg Ile 195 200 205			624
GCT CCA GAC ACA GCA AAC AAA GGA CTG ATA TCT GGT ATC TGT GGT AAT Ala Pro Asp Thr Ala Asn Lys Gly Leu Ile Ser Gly Ile Cys Gly Asn 210 215 220			672
CTG GAG ATG AAT GAC GCT GAT GAC TTT ACT ACA GAT GCA GAT CAG CTG Leu Glu Met Asn Asp Ala Asp Asp Phe Thr Thr Asp Ala Asp Gln Leu 225 230 235 240			720
GCG ATC CAA CCC AAC ATA AAC AAA GAG TTC GAC GGC TGC CCA TTC TAT Ala Ile Gln Pro Asn Ile Asn Lys Glu Phe Asp Gly Cys Pro Phe Tyr 245 250 255			768
GGC AAT CCT TCT GAT ATC GAA TAC TGC AAA GGT CTG ATG GAG CCA TAC Gly Asn Pro Ser Asp Ile Glu Tyr Cys Lys Gly Leu Met Glu Pro Tyr 260 265 270			816
AGA GCT GTA TGT CGT AAC AAT ATC AAC TTC TAC TAT TAC ACT CTA TCC Arg Ala Val Cys Arg Asn Asn Ile Asn Phe Tyr Tyr Tyr Thr Leu Ser 275 280 285			864

-11/43-

TGT	GCC	TTC	GCT	TAC	TGT	ATG	GGA	GGA	GAA	GAA	AGA	GCT	AAA	CAC	GTC	912
Cys	Ala	Phe	Ala	Tyr	Cys	Met	Gly	Gly	Glu	Glu	Arg	Ala	Lys	His	Val	
	290					295					300					
CTT	TTC	GAC	TAT	GTT	GAG	ACA	TGC	GCT	GCG	CCG	GAA	ACG	AGA	GGA	ACG	960
Leu	Phe	Asp	Tyr	Val	Glu	Thr	Cys	Ala	Ala	Pro	Glu	Thr	Arg	Gly	Thr	
305					310					315					320	
TGT	GTT	TTA	TCA	GGA	CAT	ACT	TTC	TAT	GAC	ACA	TTC	GAC	AAA	GCA	AGA	1008
Cys	Val	Leu	Ser	Gly	His	Thr	Phe	Tyr	Asp	Thr	Phe	Asp	Lys	Ala	Arg	
				325					330					335		
TAT	CAA	TTC	CAG	GGC	CCA	TGC	AAG	GAG	ATT	CTG	ATG	GCC	GCA	GAC	TGT	1056
Tyr	Gln	Phe	Gln	Gly	Pro	Cys	Lys	Glu	Ile	Leu	Met	Ala	Ala	Asp	Cys	
			340					345			—		350			
TAC	TGG	AAC	ACA	TGG	GAT	GTA	AAG	GTT	TCA	CAT	AGA	GAC	GTC	GAA	TCA	1104
Tyr	Trp	Asn	Thr	Trp	Asp	Val	Lys	Val	Ser	His	Arg	Asp	Val	Glu	Ser	
		355					360					365				
TAC	ACT	GAG	GTA	GAG	AAA	GTA	ACA	ATC	AGG	AAA	CAG	TCA	ACT	GTA	GTA	1152
Tyr	Thr	Glu	Val	Glu	Lys	Val	Thr	Ile	Arg	Lys	Gln	Ser	Thr	Val	Val	
	370					375					380					
GAT	CTC	ATT	GTG	GAT	GGC	AAG	CAG	GTC	AAG	GTT	GGA	GGA	GTG	GAT	GTA	1200
Asp	Leu	Ile	Val	Asp	Gly	Lys	Gln	Val	Lys	Val	Gly	Gly	Val	Asp	Val	
385					390					395					400	
TCT	ATC	CCG	TAC	AGC	TCT	GAG	AAC	ACT	TCC	ATA	TAC	TGG	CAG	GAT	GGA	1248
Ser	Ile	Pro	Tyr	Ser	Ser	Glu	Asn	Thr	Ser	Ile	Tyr	Trp	Gln	Asp	Gly	
				405					410					415		
GAC	ATC	CTG	ACG	ACG	GCC	ATC	CTA	CCT	GAA	GCT	CTT	GTC	GTT	AAG	TTC	1296
Asp	Ile	Leu	Thr	Thr	Ala	Ile	Leu	Pro	Glu	Ala	Leu	Val	Val	Lys	Phe	
			420					425					430			
AAC	TTT	AAG	CAG	CTC	CTT	GTA	GTT	CAT	ATC	AGA	GAT	CCA	TTC	GAT	GCA	1344
Asn	Phe	Lys	Gln	Leu	Leu	Val	Val	His	Ile	Arg	Asp	Pro	Phe	Asp	Ala	
		435					440					445				
AAG	ACA	TGC	GGC	ATA	TGT	GGT	AAC	TAT	AAT	CAA	GAT	TCA	ACT	GAT	GAT	1392
Lys	Thr	Cys	Gly	Ile	Cys	Gly	Asn	Tyr	Asn	Gln	Asp	Ser	Thr	Asp	Asp	
	450					455					460					
TTC	TTT	GAC	GCA	GAA	GGA	GCA	TGC	GCT	CTA	ACC	CCC	AAC	CCC	CCA	GGA	1440
Phe	Phe	Asp	Ala	Glu	Gly	Ala	Cys	Ala	Leu	Thr	Pro	Asn	Pro	Pro	Gly	
465					470				475						480	
TGT	ACA	GAG	GAA	CAG	AAA	CCA	GAA	GCT	GAG	CGA	CTT	TGC	AAT	AAT	CTC	1488
Cys	Thr	Glu	Glu	Gln	Lys	Pro	Glu	Ala	Glu	Arg	Leu	Cys	Asn	Asn	Leu	
				485					490					495		
TTT	GAT	TCT	TCT	ATC	GAC	GAG	AAA	TGT	AAT	GTC	TGC	TAC	AAG	CCT	GAC	1536
Phe	Asp	Ser	Ser	Ile	Asp	Glu	Lys	Cys	Asn	Val	Cys	Tyr	Lys	Pro	Asp	
				500				505					510			
CGG	ATT	GCC	CGA	TGT	ATG	TAC	GAG	TAT	TGC	CTG	AGG	GGA	CAA	CAA	GGA	1584
Arg	Ile	Ala	Arg	Cys	Met	Tyr	Glu	Tyr	Cys	Leu	Arg	Gly	Gln	Gln	Gly	
		515					520					525				
TTT	TGT	GAC	CAT	GCT	TGG	GAG	TTC	AAG	AAA	GAA	TGC	TAC	ATA	AAA	CAT	1632
Phe	Cys	Asp	His	Ala	Trp	Glu	Phe	Lys	Lys	Glu	Cys	Tyr	Ile	Lys	His	

-12/43-

530	535	540	
GGA GAC ACT CTA GAA GTA CCA CCT GAA TGT CAA TAA ACGTACAAAG			1678
Gly Asp Thr Leu Glu Val Pro Pro Glu Cys Gln			
545	550	555	
ATACAGAAGC TAAGGCTACT ACAGCAGAAG ATAAAAAAGA AACTGTAGTT CCTTCAAAAA			1738
CCGTGTATTT TATGTACTCA TTGTTTAATT AGAGCAAAT AAATTGTTAT TATCATAACT			1798
TAAACTAAAA AAAAAAAAAA AA			1820

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 958 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 115...702

(D) OTHER INFORMATION: apoaeguorin-encoding gene

(x) PUBLICATION INFORMATION:

PATENT NO.: 5,093,240

(A) AUTHORS: Inouye *et al.*

(C) JOURNAL: Proc. Natl. Acad. Sci. U.S.A.

(D) VOLUME: 82

(F) PAGES: 3154-3158

(G) DATE: (1985)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGGGGGGGGG	GGGGGGGGGG	GGGGGGGGGG	GGGAATGCAA	TTCATCTTTG	CATCAAAGAA	60
TTACATCAAA	TCTCTAGTTG	ATCAACTAAA	TTGTCTCGAC	AACAACAAGC	AAAC ATG	117
					Met	
					1	
ACA AGC AAA CAA TAC TCA GTC AAG CTT ACA TCA GAC TTC GAC AAC CCA						165
Thr Ser Lys Gln Tyr Ser Val Lys Leu Thr Ser Asp Phe Asp Asn Pro						
5		10		15		
AGA TGG ATT GGA CGA CAC AAG CAT ATG TTC AAT TTC CTT GAT GTC AAC						213
Arg Trp Ile Gly Arg His Lys His Met Phe Asn Phe Leu Asp Val Asn						
20		25		30		
CAC AAT GGA AAA ATC TCT CTT GAC GAG ATG GTC TAC AAG GCA TCT GAT						261
His Asn Gly Lys Ile Ser Leu Asp Glu Met Val Tyr Lys Ala Ser Asp						
35		40		45		
ATT GTC ATC AAT AAC CTT GGA GCA ACA CCT GAG CAA GCC AAA CGA CAC						309
Ile Val Ile Asn Asn Leu Gly Ala Thr Pro Glu Gln Ala Lys Arg His						
50		55		60		65

-13/43-

AAA GAT GCT GTA GAA GCC TTC TTC GGA GGA GCT GGA ATG AAA TAT GGT	357
Lys Asp Ala Val Glu Ala Phe Phe Gly Gly Ala Gly Met Lys Tyr Gly	
70 75 80	
GTG GAA ACT GAT TGG CCT GCA TAT ATT GAA GGA TGG AAA AAA TTG GCT	405
Val Glu Thr Asp Trp Pro Ala Tyr Ile Glu Gly Trp Lys Lys Leu Ala	
85 90 95	
ACT GAT GAA TTG GAG AAA TAC GCC AAA AAC GAA CCA ACG CTC ATC CGT	453
Thr Asp Glu Leu Glu Lys Tyr Ala Lys Asn Glu Pro Thr Leu Ile Arg	
100 105 110	
ATA TGG GGT GAT GCT TTG TTT GAT ATC GTT GAC AAA GAT CAA AAT GGA	501
Ile Trp Gly Asp Ala Leu Phe Asp Ile Val Asp Lys Asp Gln Asn Gly	
115 120 125	
GCC ATT ACA CTG GAT GAA TGG AAA GCA TAC ACC AAA GCT GCT GGT ATC	549
Ala Ile Thr Leu Asp Glu Trp Lys Ala Tyr Thr Lys Ala Ala Gly Ile	
130 135 140 145	
ATC CAA TCA TCA GAA GAT TGC GAG GAA ACA TTC AGA GTG TGC GAT ATT	597
Ile Gln Ser Ser Glu Asp Cys Glu Glu Thr Phe Arg Val Cys Asp Ile	
150 155 160	
GAT GAA AGT GGA CAA CTC GAT GTT GAT GAG ATG ACA AGA CAA CAT TTA	645
Asp Glu Ser Gly Gln Leu Asp Val Asp Glu Met Thr Arg Gln His Leu	
165 170 175	
GGA TTT TGG TAC ACC ATG GAT CCT GCT TGC GAA AAG CTC TAC GGT GGA	693
Gly Phe Trp Tyr Thr Met Asp Pro Ala Cys Glu Lys Leu Tyr Gly Gly	
180 185 190	
GCT GTC CCC TAAGAAGCTC TACGGTGGTG ATGCACCCTA GGAAGATGAT GTGATTTTGA	752
Ala Val Pro	
195	
ATAAAACACT GATGAATTCA ATCAAAATTT TCCAAATTTT TGAACGATTT CAATCGTTTG	812
TGTTGATTTT TGTAATTAGG AACAGATTAA ATCGAATGAT TAGTTGTTTT TTTAATCAAC	872
AGAACTTACA AATCGAAAAA GTAAAAAAA AAAAAAAA AAAAAAAA AAAAAAAA	932
AAAAAAA AAAAAAAA AAAAAA	958

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 591 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 1...588

(D) OTHER INFORMATION: Recombinant Aequorin AEQ1

(x) PUBLICATION INFORMATION:

-14/43-

(A) AUTHORS: Prasher et al.
 (B) TITLE: Sequence Comparisons of Complementary
 DNAs Encoding Aequorin Isotypes
 (C) JOURNAL: Biochemistry
 (D) VOLUME: 26
 (F) PAGES: 1326-1332
 (G) DATE: 1987

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATG ACC AGC GAA CAA TAC TCA GTC AAG CTT ACA CCA GAC TTC GAC AAC	48
Met Thr Ser Glu Gln Tyr Ser Val Lys Leu Thr Pro Asp Phe Asp Asn	
1 5 10 15	
CCA AAA TGG ATT GGA CGA CAC AAG CAC ATG TTT AAT TTT CTT GAT GTC	96
Pro Lys Trp Ile Gly Arg His Lys His Met Phe Asn Phe Leu Asp Val	
20 25 30	
AAC CAC AAT GGA AGG ATC TCT CTT GAC GAG ATG GTC TAC AAG GCG TCC	144
Asn His Asn Gly Arg Ile Ser Leu Asp Glu Met Val Tyr Lys Ala Ser	
35 40 45	
GAT ATT GTT ATA AAC AAT CTT GGA GCA ACA CCT GAA CAA GCC AAA CGT	192
Asp Ile Val Ile Asn Asn Leu Gly Ala Thr Pro Glu Gln Ala Lys Arg	
50 55 60	
CAC AAA GAT GCT GTA GAA GCC TTC TTC GGA GGA GCT GGA ATG AAA TAT	240
His Lys Asp Ala Val Glu Ala Phe Phe Gly Gly Ala Gly Met Lys Tyr	
65 70 75 80	
GGT GTA GAA ACT GAA TGG CCT GAA TAC ATC GAA GGA TGG AAA AGA CTG	288
Gly Val Glu Thr Glu Trp Pro Glu Tyr Ile Glu Gly Trp Lys Arg Leu	
85 90 95	
GCT TCC GAG GAA TTG AAA AGG TAT TCA AAA AAC CAA ATC ACA CTT ATT	336
Ala Ser Glu Glu Leu Lys Arg Tyr Ser Lys Asn Gln Ile Thr Leu Ile	
100 105 110	
CGT TTA TGG GGT GAT GCA TTG TTC GAT ATC ATT GAC AAA GAC CAA AAT	384
Arg Leu Trp Gly Asp Ala Leu Phe Asp Ile Ile Asp Lys Asp Gln Asn	
115 120 125	
GGA GCT ATT TCA CTG GAT GAA TGG AAA GCA TAC ACC AAA TCT GAT GGC	432
Gly Ala Ile Ser Leu Asp Glu Trp Lys Ala Tyr Thr Lys Ser Asp Gly	
130 135 140	
ATC ATC CAA TCG TCA GAA GAT TGC GAG GAA ACA TTC AGA GTG TGC GAT	480
Ile Ile Gln Ser Ser Glu Asp Cys Glu Glu Thr Phe Arg Val Cys Asp	
145 150 155 160	
ATT GAT GAA AGT GGA CAG CTC GAT GTT GAT GAG ATG ACA AGA CAA CAT	528
Ile Asp Glu Ser Gly Gln Leu Asp Val Asp Glu Met Thr Arg Gln His	
165 170 175	
TTA GGA TTT TGG TAC ACC ATG GAT CCT GCT TGC GAA AAG CTC TAC GGT	576
Leu Gly Phe Trp Tyr Thr Met Asp Pro Ala Cys Glu Lys Leu Tyr Gly	
180 185 190	
GGA GCT GTC CCC TAA	591
Gly Ala Val Pro *	
195	

-15/43-

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 591 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 1...588

(D) OTHER INFORMATION: Recombinant Aequorin AEQ2

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Prasher et al.

(B) TITLE: Sequence Comparisons of Complementary DNAs Encoding Aequorin Isotypes

(C) JOURNAL: Biochemistry

(D) VOLUME: 26

(F) PAGES: 1326-1332

(G) DATE: 1987

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATG ACC AGC GAA CAA TAC TCA GTC AAG CTT ACA TCA GAC TTC GAC AAC	48
Met Thr Ser Glu Gln Tyr Ser Val Lys Leu Thr Ser Asp Phe Asp Asn	
1 5 10 15	
CCA AGA TGG ATT GGA CGA CAC AAG CAT ATG TTC AAT TTC CTT GAT GTC	96
Pro Arg Trp Ile Gly Arg His Lys His Met Phe Asn Phe Leu Asp Val	
20 25 30	
AAC CAC AAT GGA AAA ATC TCT CTT GAC GAG ATG GTC TAC AAG GCA TCT	144
Asn His Asn Gly Lys Ile Ser Leu Asp Glu Met Val Tyr Lys Ala Ser	
35 40 45	
GAT ATT GTC ATC AAT AAC CTT GGA GCA ACA CCT GAG CAA GCC AAA CGA	192
Asp Ile Val Ile Asn Asn Leu Gly Ala Thr Pro Glu Gln Ala Lys Arg	
50 55 60	
CAC AAA GAT GCT GTA GAA GCC TTC TTC GGA GGA GCT GGA ATG AAA TAT	240
His Lys Asp Ala Val Glu Ala Phe Phe Gly Gly Ala Gly Met Lys Tyr	
65 70 75 80	
GGT GTG GAA ACT GAT TGG CCT GCA TAT ATT GAA GGA TGG AAA AAA TTG	288
Gly Val Glu Thr Asp Trp Pro Ala Tyr Ile Glu Gly Trp Lys Lys Leu	
85 90 95	
GCT ACT GAT GAA TTG GAG AAA TAC GCC AAA AAC GAA CCA ACG CTC ATC	336
Ala Thr Asp Glu Leu Glu Lys Tyr Ala Lys Asn Glu Pro Thr Leu Ile	
100 105 110	
CGT ATA TGG GGT GAT GCT TTG TTC GAT ATC GTT GAC AAA GAT CAA AAT	384
Arg Ile Trp Gly Asp Ala Leu Phe Asp Ile Val Asp Lys Asp Gln Asn	

-16/43-

115	120	125	
GGA GCC ATT ACA CTG GAT GAA TGG AAA GCA TAC ACC AAA GCT GCT GGT			432
Gly Ala Ile Thr Leu Asp Glu Trp Lys Ala Tyr Thr Lys Ala Ala Gly			
130	135	140	
ATC ATC CAA TCA TCA GAA GAT TGC GAG GAA ACA TTC AGA GTG TGC GAT			480
Ile Ile Gln Ser Ser Glu Asp Cys Glu Glu Thr Phe Arg Val Cys Asp			
145	150	155	160
ATT GAT GAA AGT GGA CAA CTC GAT GTT GAT GAG ATG ACA AGA CAA CAT			528
Ile Asp Glu Ser Gly Gln Leu Asp Val Asp Glu Met Thr Arg Gln His			
	165	170	175
TTA GGA TTT TGG TAC ACC ATG GAT CCT GCT TGC GAA AAG CTC TAC GGT			576
Leu Gly Phe Trp Tyr Thr Met Asp Pro Ala Cys Glu Lys Leu Tyr Gly			
	180	185	190
GGA GCT GTC CCC TAA			591
Gly Ala Val Pro *			
195			

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 591 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 1...588

(D) OTHER INFORMATION: Recombinant Aequorin AEQ3

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Prasher et al.

(B) TITLE: Sequence Comparisons of Complementary DNAs Encoding Aequorin Isoforms

(C) JOURNAL: Biochemistry

(D) VOLUME: 26

(F) PAGES: 1326-1332

(G) DATE: 1987

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATG ACC AGC GAA CAA TAC TCA GTC AAG CTT ACA TCA GAC TTC GAC AAC	48
Met Thr Ser Glu Gln Tyr Ser Val Lys Leu Thr Ser Asp Phe Asp Asn	
1	5
CCA AGA TGG ATT GGA CGA CAC AAG CAT ATG TTC AAT TTC CTT GAT GTC	96
Pro Arg Trp Ile Gly Arg His Lys His Met Phe Asn Phe Leu Asp Val	
20	25
	30

-17/43-

AAC CAC AAT GGA AAA ATC TCT CTT GAC GAG ATG GTC TAC AAG GCA TCT	144
Asn His Asn Gly Lys Ile Ser Leu Asp Glu Met Val Tyr Lys Ala Ser	
35 40 45	
GAT ATT GTC ATC AAT AAC CTT GGA GCA ACA CCT GAG CAA GCC AAA CGA	192
Asp Ile Val Ile Asn Asn Leu Gly Ala Thr Pro Glu Gln Ala Lys Arg	
50 55 60	
CAC AAA GAT GCT GTA GGA GAC TTC TTC GGA GGA GCT GGA ATG AAA TAT	240
His Lys Asp Ala Val Gly Asp Phe Phe Gly Gly Ala Gly Met Lys Tyr	
65 70 75 80	
GGT GTG GAA ACT GAT TGG CCT GCA TAC ATT GAA GGA TGG AAA AAA TTG	288
Gly Val Glu Thr Asp Trp Pro Ala Tyr Ile Glu Gly Trp Lys Lys Leu	
85 90 95	
GCT ACT GAT GAA TTG GAG AAA TAC GCC AAA AAC GAA CCA ACG CTC ATC	336
Ala Thr Asp Glu Leu Glu Lys Tyr Ala Lys Asn Glu Pro Thr Leu Ile	
100 105 110	
CGT ATA TGG GGT GAT GCT TTG TTC GAT ATC GTT GAC AAA GAT CAA AAT	384
Arg Ile Trp Gly Asp Ala Leu Phe Asp Ile Val Asp Lys Asp Gln Asn	
115 120 125	
GGA GCC ATT ACA CTG GAT GAA TGG AAA GCA TAC ACC AAA GCT GCT GGT	432
Gly Ala Ile Thr Leu Asp Glu Trp Lys Ala Tyr Thr Lys Ala Ala Gly	
130 135 140	
ATC ATC CAA TCA TCA GAA GAT TGC GAG GAA ACA TTC AGA GTG TGC GAT	480
Ile Ile Gln Ser Ser Glu Asp Cys Glu Glu Thr Phe Arg Val Cys Asp	
145 150 155 160	
ATT GAT GAA AAT GGA CAA CTC GAT GTT GAT GAG ATG ACA AGA CAA CAT	528
Ile Asp Glu Asn Gly Gln Leu Asp Val Asp Glu Met Thr Arg Gln His	
165 170 175	
TTA GGA TTT TGG TAC ACC ATG GAT CCT GCT TGC GAA AAG CTC TAC GGT	576
Leu Gly Phe Trp Tyr Thr Met Asp Pro Ala Cys Glu Lys Leu Tyr Gly	
180 185 190	
GGA GCT GTC CCC TAA	591
Gly Ala Val Pro *	
195	

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 567 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 1...567

-18/43-

(D) OTHER INFORMATION: Aequorin photoprotein

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Charbonneau et al.
 (B) TITLE: Amino acid sequence of the calcium-dependent photoprotein aequorin
 (C) JOURNAL: Am. Chem. Soc.
 (D) VOLUME: 24
 (E) ISSUE: 24
 (F) PAGES: 6762-6771
 (G) DATE: 1985

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GTC AAG CTT ACA CCA GAC TTC GAC AAC CCA AAA TGG ATT GGA CGA CAG	48
Val Lys Leu Thr Pro Asp Phe Asp Asn Pro Lys Trp Ile Gly Arg His	
1 5 10 15	
AAG CAC ATG TTT AAT TTT CTT GAT GTC AAC CAC AAT GGA AGG ATC TCT	96
Lys His Met Phe Asn Phe Leu Asp Val Asn His Asn Gly Arg Ile Ser	
20 25 30	
CTT GAC GAG ATG GTC TAC AAG GCG TCC GAT ATT GTT ATA AAC AAT CTT	144
Leu Asp Glu Met Val Tyr Lys Ala Ser Asp Ile Val Ile Asn Asn Leu	
35 40 45	
GGA GCA ACA CCT GAA CAA GCC AAA CGT CAC AAA GAT GCT GTA GAA GCC	192
Gly Ala Thr Pro Glu Gln Ala Lys Arg His Lys Asp Ala Val Glu Ala	
50 55 60	
TTC TTC GGA GGA GCT GCA ATG AAA TAT GGT GTA GAA ACT GAA TGG CCT	240
Phe Phe Gly Gly Ala Ala Met Lys Tyr Gly Val Glu Thr Glu Trp Pro	
65 70 75 80	
GAA TAC ATC GAA GGA TGG AAA AGA CTG GCT TCC GAG GAA TTG AAA AGG	288
Glu Tyr Ile Glu Gly Trp Lys Arg Leu Ala Ser Glu Glu Leu Lys Arg	
85 90 95	
TAT TCA AAA AAC CAA ATC ACA CTT ATT CGT TTA TGG GGT GAT GCA TTG	336
Tyr Ser Lys Asn Gln Ile Thr Leu Ile Arg Leu Trp Gly Asp Ala Leu	
100 105 110	
TTC GAT ATC ATT GAC AAA GAC CAA AAT GGA GCT ATT TCA CTG GAT GAA	384
Phe Asp Ile Ile Asp Lys Asp Gln Asn Gly Ala Ile Ser Leu Asp Glu	
115 120 125	
TGG AAA GCA TAC ACC AAA TCT GCT GGC ATC ATC CAA TCG TCA GAA GAT	432
Trp Lys Ala Tyr Thr Lys Ser Ala Gly Ile Ile Gln Ser Ser Glu Asp	
130 135 140	
TGC GAG GAA ACA TTC AGA GTG TGC GAT ATT GAT GAA AGT GGA CAG CTC	480
Cys Glu Glu Thr Phe Arg Val Cys Asp Ile Asp Glu Ser Gly Gln Leu	
145 150 155 160	
GAT GTT GAT GAG ATG ACA AGA CAA CAT TTA GGA TTT TGG TAC ACC ATG	528
Asp Val Asp Glu Met Thr Arg Gln His Leu Gly Phe Trp Tyr Thr Met	
165 170 175	
GAT CCT GCT TGC GAA AAG CTC TAC GGT GGA GCT GTC CCC	567
Asp Pro Ala Cys Glu Lys Leu Tyr Gly Gly Ala Val Pro	

-19/43-

180

185

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 588 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 1...588

(D) OTHER INFORMATION: Aequorin mutant w/increased bioluminescence activity

(x) PUBLICATION INFORMATION:

PATENT NO.: 5,360,728

(K) RELEVANT RESIDUES IN SEQ ID NO: 10:

Asp 124 changed to Ser

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATG ACC AGC GAA CAA TAC TCA GTC AAG CTT ACA CCA GAC TTC GAC AAC	48
Met Thr Ser Glu Gln Tyr Ser Val Lys Leu Thr Pro Asp Phe Asp Asn	
1 5 10 15	
CCA AAA TGG ATT GGA CGA CAC AAG CAC ATG TTT AAT TTT CTT GAT GTC	96
Pro Lys Trp Ile Gly Arg His Lys His Met Phe Asn Phe Leu Asp Val	
20 25 30	
AAC CAC AAT GGA AGG ATC TCT CTT GAC GAG ATG GTC TAC AAG GCG TCC	144
Asn His Asn Gly Arg Ile Ser Leu Asp Glu Met Val Tyr Lys Ala Ser	
35 40 45	
GAT ATT GTT ATA AAC AAT CTT GGA GCA ACA CCT GAA CAA GCC AAA CGT	192
Asp Ile Val Ile Asn Asn Leu Gly Ala Thr Pro Glu Gln Ala Lys Arg	
50 55 60	
CAC AAA GAT GCT GTA GAA GCC TTC TTC GGA GGA GCT GCA ATG AAA TAT	240
His Lys Asp Ala Val Glu Ala Phe Phe Gly Gly Ala Ala Met Lys Tyr	
65 70 75 80	
GGT GTA GAA ACT GAA TGG CCT GAA TAC ATC GAA GGA TGG AAA AGA CTG	288
Gly Val Glu Thr Glu Trp Pro Glu Tyr Ile Glu Gly Trp Lys Arg Leu	
85 90 95	
GCT TCC GAG GAA TTG AAA AGG TAT TCA AAA AAC CAA ATC ACA CTT ATT	336
Ala Ser Glu Glu Leu Lys Arg Tyr Ser Lys Asn Gln Ile Thr Leu Ile	
100 105 110	
CGT TTA TGG GGT GAT GCA TTG TTC GAT ATC ATT TCC AAA GAC CAA AAT	384
Arg Leu Trp Gly Asp Ala Leu Phe Asp Ile Ile Ser Lys Asp Gln Asn	

-20/43-

115	120	125	
GGA GCT ATT TCA CTG GAT GAA TGG AAA GCA TAC ACC AAA TCT GCT GGC			432
Gly Ala Ile Ser Leu Asp Glu Trp Lys Ala Tyr Thr Lys Ser Ala Gly			
130	135	140	
ATC ATC CAA TCG TCA GAA GAT TGC GAG GAA ACA TTC AGA GTG TGC GAT			480
Ile Ile Gln Ser Ser Glu Asp Cys Glu Glu Thr Phe Arg Val Cys Asp			
145	150	155	160
ATT GAT GAA AGT GGA CAG CTC GAT GTT GAT GAG ATG ACA AGA CAA CAT			528
Ile Asp Glu Ser Gly Gln Leu Asp Val Asp Glu Met Thr Arg Gln His			
165	170	175	
TTA GGA TTT TGG TAC ACC ATG GAT CCT GCT TGC GAA AAG CTC TAC GGT			576
Leu Gly Phe Trp Tyr Thr Met Asp Pro Ala Cys Glu Lys Leu Tyr Gly			
180	185	190	
GGA GCT GTC CCC			588
Gly Ala Val Pro			
195			

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 588 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 1...588

(D) OTHER INFORMATION: Recombinant site-directed Aequorin mutant
w/increased biolum. activity

(x) PUBLICATION INFORMATION:

PATENT NO.: 5,360,728

(K) RELEVANT RESIDUES IN SEQ ID NO: 11:

Glu 135 changed to Ser

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATG ACC AGC GAA CAA TAC TCA GTC AAG CTT ACA CCA GAC TTC GAC AAC	48
Met Thr Ser Glu Gln Tyr Ser Val Lys Leu Thr Pro Asp Phe Asp Asn	
1	5
CCA AAA TGG ATT GGA CGA CAC AAG CAC ATG TTT AAT TTT CTT GAT GTC	96
Pro Lys Trp Ile Gly Arg His Lys His Met Phe Asn Phe Leu Asp Val	
20	25
AAC CAC AAT GGA AGG ATC TCT CTT GAC GAG ATG GTC TAC AAG GCG TCC	144
Asn His Asn Gly Arg Ile Ser Leu Asp Glu Met Val Tyr Lys Ala Ser	

-21/43-

35					40					45						
GAT	ATT	GTT	ATA	AAC	AAT	CTT	GGA	GCA	ACA	CCT	GAA	CAA	GCC	AAA	CGT	192
Asp	Ile	Val	Ile	Asn	Asn	Leu	Gly	Ala	Thr	Pro	Glu	Gln	Ala	Lys	Arg	
50						55					60					
CAC	AAA	GAT	GCT	GTA	GAA	GCC	TTC	TTC	GGA	GGA	GCT	GCA	ATG	AAA	TAT	240
His	Lys	Asp	Ala	Val	Glu	Ala	Phe	Phe	Gly	Gly	Ala	Ala	Met	Lys	Tyr	
65					70				75					80		
GGT	GTA	GAA	ACT	GAA	TGG	CCT	GAA	TAC	ATC	GAA	GGA	TGG	AAA	AGA	CTG	288
Gly	Val	Glu	Thr	Glu	Trp	Pro	Glu	Tyr	Ile	Glu	Gly	Trp	Lys	Arg	Leu	
				85				90					95			
GCT	TCC	GAG	GAA	TTG	AAA	AGG	TAT	TCA	AAA	AAC	CAA	ATC	ACA	CTT	ATT	336
Ala	Ser	Glu	Glu	Leu	Lys	Arg	Tyr	Ser	Lys	Asn	Gln	Ile	Thr	Leu	Ile	
			100					105					110			
CGT	TTA	TGG	GGT	GAT	GCA	TTG	TTC	GAT	ATC	ATT	TCC	AAA	GAC	CAA	AAT	384
Arg	Leu	Trp	Gly	Asp	Ala	Leu	Phe	Asp	Ile	Ile	Ser	Lys	Asp	Gln	Asn	
		115					120					125				
GGA	GCT	ATT	TCA	CTG	GAT	TCA	TGG	AAA	GCA	TAC	ACC	AAA	TCT	GCT	GGC	432
Gly	Ala	Ile	Ser	Leu	Asp	Ser	Trp	Lys	Ala	Tyr	Thr	Lys	Ser	Ala	Gly	
	130					135					140					
ATC	ATC	CAA	TCG	TCA	GAA	GAT	TGC	GAG	GAA	ACA	TTC	AGA	GTG	TGC	GAT	480
Ile	Ile	Gln	Ser	Ser	Glu	Asp	Cys	Glu	Glu	Thr	Phe	Arg	Val	Cys	Asp	
145					150					155				160		
ATT	GAT	GAA	AGT	GGA	CAG	CTC	GAT	GTT	GAT	GAG	ATG	ACA	AGA	CAA	CAT	528
Ile	Asp	Glu	Ser	Gly	Gln	Leu	Asp	Val	Asp	Glu	Met	Thr	Arg	Gln	His	
				165				170						175		
TTA	GGA	TTT	TGG	TAC	ACC	ATG	GAT	CCT	GCT	TGC	GAA	AAG	CTC	TAC	GGT	576
Leu	Gly	Phe	Trp	Tyr	Thr	Met	Asp	Pro	Ala	Cys	Glu	Lys	Leu	Tyr	Gly	
			180					185					190			
GGA	GCT	GTC	CCC													588
Gly	Ala	Val	Pro													
			195													

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 588 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 1...588

(D) OTHER INFORMATION: Recombinant site-directed Aequorin mutant w/increased biolum. activity

-22/43-

(x) PUBLICATION INFORMATION:
PATENT NO.: 5,360,728

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATG ACC AGC GAA CAA TAC TCA GTC AAG CTT ACA CCA GAC TTC GAC AAC	48
Met Thr Ser Glu Gln Tyr Ser Val Lys Leu Thr Pro Asp Phe Asp Asn	
1 5 10 15	
CCA AAA TGG ATT GGA CGA CAC AAG CAC ATG TTT AAT TTT CTT GAT GTC	96
Pro Lys Trp Ile Gly Arg His Lys His Met Phe Asn Phe Leu Asp Val	
20 25 30	
AAC CAC AAT GGA AGG ATC TCT CTT GAC GAG ATG GTC TAC AAG GCG TCC	144
Asn His Asn Gly Arg Ile Ser Leu Asp Glu Met Val Tyr Lys Ala Ser	
35 40 45	
GAT ATT GTT ATA AAC AAT CTT GGA GCA ACA CCT GAA CAA GCC AAA CGT	192
Asp Ile Val Ile Asn Asn Leu Gly Ala Thr Pro Glu Gln Ala Lys Arg	
50 55 60	
CAC AAA GAT GCT GTA GAA GCC TTC TTC GGA GGA GCT GCA ATG AAA TAT	240
His Lys Asp Ala Val Glu Ala Phe Phe Gly Gly Ala Ala Met Lys Tyr	
65 70 75 80	
GGT GTA GAA ACT GAA TGG CCT GAA TAC ATC GAA GGA TGG AAA AGA CTG	288
Gly Val Glu Thr Glu Trp Pro Glu Tyr Ile Glu Gly Trp Lys Arg Leu	
85 90 95	
GCT TCC GAG GAA TTG AAA AGG TAT TCA AAA AAC CAA ATC ACA CTT ATT	336
Ala Ser Glu Glu Leu Lys Arg Tyr Ser Lys Asn Gln Ile Thr Leu Ile	
100 105 110	
CGT TTA TGG GGT GAT GCA TTG TTC GAT ATC ATT TCC AAA GAC CAA AAT	384
Arg Leu Trp Gly Asp Ala Leu Phe Asp Ile Ile Ser Lys Asp Gln Asn	
115 120 125	
GCA GCT ATT TCA CTG GAT GAA TGG AAA GCA TAC ACC AAA TCT GCT GGC	432
Ala Ala Ile Ser Leu Asp Glu Trp Lys Ala Tyr Thr Lys Ser Ala Gly	
130 135 140	
ATC ATC CAA TCG TCA GAA GAT TGC GAG GAA ACA TTC AGA GTG TGC GAT	480
Ile Ile Gln Ser Ser Glu Asp Cys Glu Glu Thr Phe Arg Val Cys Asp	
145 150 155 160	
ATT GAT GAA AGT GGA CAG CTC GAT GTT GAT GAG ATG ACA AGA CAA CAT	528
Ile Asp Glu Ser Gly Gln Leu Asp Val Asp Glu Met Thr Arg Gln His	
165 170 175	
TTA GGA TTT TGG TAC ACC ATG GAT CCT GCT TGC GAA AAG CTC TAC GGT	576
Leu Gly Phe Trp Tyr Thr Met Asp Pro Ala Cys Glu Lys Leu Tyr Gly	
180 185 190	
GGA GCT GTC CCC	588
Gly Ala Val Pro	
195	

-23/43-

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 567 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
- (B) LOCATION: 1...567
- (D) OTHER INFORMATION: Recombinant apoaeguorin (AQUALITE®)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTC	AAG	CTT	ACA	CCA	GAC	TTC	GAC	AAC	CCA	AAA	TGG	ATT	GGA	CGA	CAC	48
Val	Lys	Leu	Thr	Pro	Asp	Phe	Asp	Asn	Pro	Lys	Trp	Ile	Gly	Arg	His	
1				5				10						15		
AAG	CAC	ATG	TTT	AAT	TTT	CTT	GAT	GTC	AAC	CAC	AAT	GGA	AGG	ATC	TCT	96
Lys	His	Met	Phe	Asn	Phe	Leu	Asp	Val	Asn	His	Asn	Gly	Arg	Ile	Ser	
			20					25					30			
CTT	GAC	GAG	ATG	GTC	TAC	AAG	GCG	TCC	GAT	ATT	GTT	ATA	AAC	AAT	CTT	144
Leu	Asp	Glu	Met	Val	Tyr	Lys	Ala	Ser	Asp	Ile	Val	Ile	Asn	Asn	Leu	
		35					40					45				
GGA	GCA	ACA	CCT	GAA	CAA	GCC	AAA	CGT	CAC	AAA	GAT	GCT	GTA	GAA	GCC	192
Gly	Ala	Thr	Pro	Glu	Gln	Ala	Lys	Arg	His	Lys	Asp	Ala	Val	Glu	Ala	
	50					55					60					
TTC	TTC	GGA	GGA	GCT	GGA	ATG	AAA	TAT	GGT	GTA	GAA	ACT	GAA	TGG	CCT	240
Phe	Phe	Gly	Gly	Ala	Gly	Met	Lys	Tyr	Gly	Val	Glu	Thr	Glu	Trp	Pro	
65				70					75						80	
GAA	TAC	ATC	GAA	GGA	TGG	AAA	AAA	CTG	GCT	TCC	GAG	GAA	TTG	AAA	AGG	288
Glu	Tyr	Ile	Glu	Gly	Trp	Lys	Lys	Leu	Ala	Ser	Glu	Glu	Leu	Lys	Arg	
			85					90						95		
TAT	TCA	AAA	AAC	CAA	ATC	ACA	CTT	ATT	CGT	TTA	TGG	GGT	GAT	GCA	TTG	336
Tyr	Ser	Lys	Asn	Gln	Ile	Thr	Leu	Ile	Arg	Leu	Trp	Gly	Asp	Ala	Leu	
			100					105					110			
TTC	GAT	ATC	ATT	GAC	AAA	GAC	CAA	AAT	GGA	GCT	ATT	CTG	TCA	GAT	GAA	384
Phe	Asp	Ile	Ile	Asp	Lys	Asp	Gln	Asn	Gly	Ala	Ile	Leu	Ser	Asp	Glu	
		115					120					125				
TGG	AAA	GCA	TAC	ACC	AAA	TCT	GAT	GGC	ATC	ATC	CAA	TCG	TCA	GAA	GAT	432
Trp	Lys	Ala	Tyr	Thr	Lys	Ser	Asp	Gly	Ile	Ile	Gln	Ser	Ser	Glu	Asp	
	130					135					140					
TGC	GAG	GAA	ACA	TTC	AGA	GTG	TGC	GAT	ATT	GAT	GAA	AGT	GGA	CAG	CTC	480
Cys	Glu	Glu	Thr	Phe	Arg	Val	Cys	Asp	Ile	Asp	Glu	Ser	Gly	Gln	Leu	
145					150					155					160	
GAT	GTT	GAT	GAG	ATG	ACA	AGA	CAA	CAT	TTA	GGA	TTT	TGG	TAC	ACC	ATG	528
Asp	Val	Asp	Glu	Met	Thr	Arg	Gln	His	Leu	Gly	Phe	Trp	Tyr	Thr	Met	
				165					170					175		

-24/43-

GAT CCT GCT TGC GAA AAG CTC TAC GGT GGA GCT GTC CCC
 Asp Pro Ala Cys Glu Lys Leu Tyr Gly Gly Ala Val Pro
 180 185

567

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 236 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(x) PUBLICATION INFORMATION:

PATENT NO.: 5,484,723

(ix) FEATURE:

- (D) OTHER INFORMATION: *Vibrio fischeri* Flavin reductase

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Met Pro Ile Asn Cys Lys Val Lys Ser Ile Glu Pro Leu Ala Cys Asn
 1 5 10 15
 Thr Phe Arg Ile Leu Leu His Pro Glu Gln Pro Val Ala Phe Lys Ala
 20 25 30
 Gly Gln Tyr Leu Thr Val Val Met Gly Glu Lys Asp Lys Arg Pro Phe
 35 40 45
 Ser Ile Ala Ser Ser Pro Cys Arg His Glu Gly Glu Ile Glu Leu His
 50 55 60
 Ile Gly Ala Ala Glu His Asn Ala Tyr Ala Gly Glu Val Val Glu Ser
 65 70 75 80
 Met Lys Ser Ala Leu Glu Thr Gly Gly Asp Ile Leu Ile Asp Ala Pro
 85 90 95
 His Gly Glu Ala Trp Ile Arg Glu Asp Ser Asp Arg Ser Met Leu Leu
 100 105 110
 Ile Ala Gly Gly Thr Gly Phe Ser Tyr Val Arg Ser Ile Leu Asp His
 115 120 125
 Cys Ile Ser Gln Gln Ile Gln Lys Pro Ile Tyr Leu Tyr Trp Gly Gly
 130 135 140
 Arg Asp Glu Cys Gln Leu Tyr Ala Lys Ala Glu Leu Glu Ser Ile Ala
 145 150 155 160
 Gln Ala His Ser His Ile Thr Phe Val Pro Val Val Glu Lys Ser Glu
 165 170 175
 Gly Trp Thr Gly Lys Thr Gly Asn Val Leu Glu Ala Val Lys Ala Asp
 180 185 190
 Phe Asn Ser Leu Ala Asp Met Asp Ile Tyr Ile Ala Gly Arg Phe Glu
 195 200 205

-25/43-

Met Ala Gly Ala Ala Arg Glu Gln Phe Thr Thr Glu Lys Gln Ala Lys
 210 215 220

Lys Glu Gln Leu Phe Gly Asp Ala Phe Ala Phe Ile
 225 230 235

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1079 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 259...975

(D) OTHER INFORMATION: *Renilla muelleri* GFP

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTTTATACAC	AAGTGTATCG	CGTATCTGCA	GACGCATCTA	GTGGGATTAT	TCGAGCGGTA	60
GTATTTACGT	CAGACCTGTC	TAATCGAAAC	CACAACAAAC	TCTTAAATA	AGCCACATTT	120
ACATAATATC	TAAGAGACGC	CTCATTTAAG	AGTAGTAAAA	ATATAATATA	TGATAGAGTA	180
TACAACTCTC	GCCTTAGACA	GACAGTGTGC	AACAGAGTAA	CTCTTGTTAA	TGCAATCGAA	240
AGCGTCAAGA	GAGATAAG	ATG AGT AAA	CAA ATA TTG	AAG AAC ACT	TGT TTA	291
	Met Ser Lys	Gln Ile Leu	Lys Asn Thr	Cys Leu		
	1	5	10			
CAA GAA GTA	ATG TCG TAT	AAA GTA AAT	CTG GAA GGA	ATT GTA AAC	AAC	339
Gln Glu Val	Met Ser Tyr	Lys Val Asn	Leu Glu Gly	Ile Val Asn	Asn	
	15	20	25			
CAT GTT TTT	ACA ATG GAG	GGT TGC GGC	AAA GGG AAT	ATT TTA TTC	GGC	387
His Val Phe	Thr Met Glu	Gly Cys Gly	Lys Gly Asn	Ile Leu Phe	Gly	
	30	35	40			
AAT CAA CTG	GTT CAG ATT	CGT GTC ACG	AAA GGG GCC	CCA CTG CCT	TTT	435
Asn Gln Leu	Val Gln Ile	Arg Val Thr	Lys Gly Ala	Pro Leu Pro	Phe	
	45	50	55			
GCA TTT GAT	ATT GTG TCA	CCA GCT TTT	CAA TAT GGC	AAC CGT ACT	TTC	483
Ala Phe Asp	Ile Val Ser	Pro Ala Phe	Gln Tyr Gly	Asn Arg Thr	Phe	
	60	65	70		75	
ACG AAA TAT	CCG AAT GAT	ATA TCA GAT	TAT TTT ATA	CAA TCA TTT	CCA	531
Thr Lys Tyr	Pro Asn Asp	Ile Ser Asp	Tyr Phe Ile	Gln Ser Phe	Pro	
	80	85	90			
GCA GGA TTT	ATG TAT GAA	CGA ACA TTA	CGT TAC GAA	GAT GGC GGA	CTT	579
Ala Gly Phe	Met Tyr Glu	Arg Thr Leu	Arg Tyr Glu	Asp Gly Gly	Leu	
	95	100	105			
GTT GAA ATT	CGT TCA GAT	ATA AAT TTA	ATA GAA GAC	AAG TTC GTC	TAC	627
Val Glu Ile	Arg Ser Asp	Ile Asn Leu	Ile Glu Asp	Lys Phe Val	Tyr	

-26/43-

110	115	120	
AGA GTG GAA TAC AAA GGT AGT AAC TTC CCA GAT GAT GGT CCC GTC ATG			675
Arg Val Glu Tyr Lys Gly Ser Asn Phe Pro Asp Asp Gly Pro Val Met			
125	130	135	
CAG AAG ACT ATC TTA GGA ATA GAG CCT TCA TTT GAA GCC ATG TAC ATG			723
Gln Lys Thr Ile Leu Gly Ile Glu Pro Ser Phe Glu Ala Met Tyr Met			
140	145	150	155
AAT AAT GGC GTC TTG GTC GGC GAA GTA ATT CTT GTC TAT AAA CTA AAC			771
Asn Asn Gly Val Leu Val Gly Glu Val Ile Leu Val Tyr Lys Leu Asn			
	160	165	170
TCT GGG AAA TAT TAT TCA TGT CAC ATG AAA ACA TTA ATG AAG TCG AAA			819
Ser Gly Lys Tyr Tyr Ser Cys His Met Lys Thr Leu Met Lys Ser Lys			
	175	180	185
GGT GTA GTA AAG GAG TTT CCT TCG TAT CAT TTT ATT CAA CAT CGT TTG			867
Gly Val Val Lys Glu Phe Pro Ser Tyr His Phe Ile Gln His Arg Leu			
	190	195	200
GAA AAG ACT TAC GTA GAA GAC GGG GGG TTC GTT GAA CAG CAT GAG ACT			915
Glu Lys Thr Tyr Val Glu Asp Gly Gly Phe Val Glu Gln His Glu Thr			
	205	210	215
GCT ATT GCT CAA ATG ACA TCT ATA GGA AAA CCA CTA GGA TCC TTA CAC			963
Ala Ile Ala Gln Met Thr Ser Ile Gly Lys Pro Leu Gly Ser Leu His			
	220	225	230
GAA TGG GTT TAA ACACAGTTAC ATTACTTTTT CCAATTCGTG TTTCATGTCA AATAAT			1021
Glu Trp Val *			
AATTTTTTAA ACAATTATCA ATGTTTTGTG ATATGTTTGT AAAAAAAAAA AAAAAAAAAA			1079

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 238 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Met	Ser	Lys	Gln	Ile	Leu	Lys	Asn	Thr	Cys	Leu	Gln	Glu	Val	Met	Ser
1				5					10					15	
Tyr	Lys	Val	Asn	Leu	Glu	Gly	Ile	Val	Asn	Asn	His	Val	Phe	Thr	Met
			20					25					30		
Glu	Gly	Cys	Gly	Lys	Gly	Asn	Ile	Leu	Phe	Gly	Asn	Gln	Leu	Val	Gln
		35				40					45				
Ile	Arg	Val	Thr	Lys	Gly	Ala	Pro	Leu	Pro	Phe	Ala	Phe	Asp	Ile	Val
	50					55				60					
Ser	Pro	Ala	Phe	Gln	Tyr	Gly	Asn	Arg	Thr	Phe	Thr	Lys	Tyr	Pro	Asn

-27/43-

65	Asp	Ile	Ser	Asp	Tyr	70	Phe	Ile	Gln	Ser	75	Phe	Pro	Ala	Gly	Phe	Met	80	Tyr
					85							90	Gly	Leu	Val	Glu	Ile	95	Arg
	Glu	Arg	Thr	Leu	Arg		Tyr	Glu	Asp	Gly									Ser
				100						105								110	
	Asp	Ile	Asn	Leu	Ile	Glu	Asp	Lys	Phe	Val	Tyr	Arg	Val	Glu	Tyr	Lys			
			115					120						125					
	Gly	Ser	Asn	Phe	Pro	Asp	Asp	Gly	Pro	Val	Met	Gln	Lys	Thr	Ile	Leu			
			130					135				140							
	Gly	Ile	Glu	Pro	Ser	Phe	Glu	Ala	Met	Tyr	Met	Asn	Asn	Gly	Val	Leu			
145						150					155							160	
	Val	Gly	Glu	Val	Ile	Leu	Val	Tyr	Lys	Leu	Asn	Ser	Gly	Lys	Tyr	Tyr			
					165					170					175				
	Ser	Cys	His	Met	Lys	Thr	Leu	Met	Lys	Ser	Lys	Gly	Val	Val	Lys	Glu			
				180					185					190					
	Phe	Pro	Ser	Tyr	His	Phe	Ile	Gln	His	Arg	Leu	Glu	Lys	Thr	Tyr	Val			
			195					200					205						
	Glu	Asp	Gly	Gly	Phe	Val	Glu	Gln	His	Glu	Thr	Ala	Ile	Ala	Gln	Met			
			210					215				220							
	Thr	Ser	Ile	Gly	Lys	Pro	Leu	Gly	Ser	Leu	His	Glu	Trp	Val					
225						230					235								

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1217 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 31...963

(D) OTHER INFORMATION: *Renilla mulleri* luciferase

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CGGCACGAGG	TTTAAGAATC	AATAAAAAAA	ATG	ACG	TCA	AAA	GTT	TAC	GAT	CCT	54					
			Met	Thr	Ser	Lys	Val	Tyr	Asp	Pro						
			1				5									
GAA	TTA	AGA	AAA	CGC	ATG	ATT	ACT	GGT	CCA	CAA	TGG	TGG	GCA	AGA	TGT	102
Glu	Leu	Arg	Lys	Arg	Met	Ile	Thr	Gly	Pro	Gln	Trp	Trp	Ala	Arg	Cys	
	10					15					20					
AAA	CAA	ATG	AAT	GTT	CTT	GAT	TCA	TTT	ATT	AAT	TAT	TAT	GAT	TCA	GAA	150
Lys	Gln	Met	Asn	Val	Leu	Asp	Ser	Phe	Ile	Asn	Tyr	Tyr	Asp	Ser	Glu	
	25				30				35					40		
AAA	CAT	GCA	GAA	AAT	GCA	GTT	ATA	TTT	TTA	CAT	GGT	AAT	GCA	GCA	TCT	198
Lys	His	Ala	Glu	Asn	Ala	Val	Ile	Phe	Leu	His	Gly	Asn	Ala	Ala	Ser	
			45					50					55			
TCT	TAT	TTA	TGG	CGT	CAT	GTT	GTA	CCA	CAT	GTT	GAA	CCA	GTG	GCG	CGA	246

-28/43-

Ser	Tyr	Leu	Trp	Arg	His	Val	Val	Pro	His	Val	Glu	Pro	Val	Ala	Arg	
			60					65					70			
TGT	ATT	ATA	CCA	GAT	CTT	ATA	GGT	ATG	GGT	AAA	TCA	GGC	AAG	TCT	GGT	294
Cys	Ile	Ile	Pro	Asp	Leu	Ile	Gly	Met	Gly	Lys	Ser	Gly	Lys	Ser	Gly	
		75					80					85				
AAT	GGT	TCC	TAT	AGA	TTA	CTA	GAT	CAT	TAC	AAA	TAT	CTT	ACT	GAA	TGG	342
Asn	Gly	Ser	Tyr	Arg	Leu	Leu	Asp	His	Tyr	Lys	Tyr	Leu	Thr	Glu	Trp	
	90					95					100					
TTC	AAA	CAT	CTT	AAT	TTA	CCA	AAG	AAG	ATC	ATT	TTT	GTC	GGT	CAT	GAT	390
Phe	Lys	His	Leu	Asn	Leu	Pro	Lys	Lys	Ile	Ile	Phe	Val	Gly	His	Asp	
105					110					115					120	
TGG	GGT	GCT	TGT	TTA	GCA	TTT	CAT	TAT	TGC	TAT	GAA	CAT	CAG	GAT	CGC	438
Trp	Gly	Ala	Cys	Leu	Ala	Phe	His	Tyr	Cys	Tyr	Glu	His	Gln	Asp	Arg	
				125					130					135		
ATC	AAA	GCA	GTT	GTT	CAT	GCT	GAA	AGT	GTA	GTA	GAT	GTG	ATT	GAA	TCG	486
Ile	Lys	Ala	Val	Val	His	Ala	Glu	Ser	Val	Val	Asp	Val	Ile	Glu	Ser	
			140					145					150			
TGG	GAC	GAA	TGG	CCT	GAT	ATT	GAA	GAA	GAT	ATT	GCT	TTG	ATT	AAA	TCT	534
Trp	Asp	Glu	Trp	Pro	Asp	Ile	Glu	Glu	Asp	Ile	Ala	Leu	Ile	Lys	Ser	
		155					160					165				
GAA	GAA	GGA	GAA	AAA	ATG	GTT	TTA	GAG	AAT	AAC	TTC	TTC	GTG	GAA	ACC	582
Glu	Glu	Gly	Glu	Lys	Met	Val	Leu	Glu	Asn	Asn	Phe	Phe	Val	Glu	Thr	
	170					175					180					
ATG	TTG	CCA	TCA	AAA	ATC	ATG	AGA	AAG	TTG	GAA	CCA	GAG	GAA	TTT	GCT	630
Met	Leu	Pro	Ser	Lys	Ile	Met	Arg	Lys	Leu	Glu	Pro	Glu	Glu	Phe	Ala	
185					190					195					200	
GCT	TAT	CTT	GAA	CCA	TTT	AAA	GAG	AAA	GGT	GAA	GTT	CGT	CGT	CCA	ACA	678
Ala	Tyr	Leu	Glu	Pro	Phe	Lys	Glu	Lys	Gly	Glu	Val	Arg	Arg	Pro	Thr	
				205					210					215		
TTA	TCA	TGG	CCT	CGT	GAA	ATC	CCT	TTG	GTA	AAA	GGT	GGT	AAA	CCG	GAT	726
Leu	Ser	Trp	Pro	Arg	Glu	Ile	Pro	Leu	Val	Lys	Gly	Gly	Lys	Pro	Asp	
			220				225						230			
GTA	GTA	GAA	ATT	GTC	AGG	AAT	TAT	AAT	GCT	TAT	CTT	CGT	GCA	AGT	CAT	774
Val	Val	Glu	Ile	Val	Arg	Asn	Tyr	Asn	Ala	Tyr	Leu	Arg	Ala	Ser	His	
		235					240					245				
GAT	TTA	CCA	AAA	ATG	TTT	ATT	GAA	TCT	GAT	CCA	GGA	TTC	TTT	TCC	AAT	822
Asp	Leu	Pro	Lys	Met	Phe	Ile	Glu	Ser	Asp	Pro	Gly	Phe	Phe	Ser	Asn	
	250					255					260					
GCT	ATT	GTT	GAA	GGT	GCT	AAG	AAA	TTC	CCT	AAT	ACT	GAA	TTT	GTT	AAA	870
Ala	Ile	Val	Glu	Gly	Ala	Lys	Lys	Phe	Pro	Asn	Thr	Glu	Phe	Val	Lys	
265					270					275					280	
GTC	AAA	GGT	CTT	CAT	TTT	TCA	CAA	GAA	GAT	GCA	CCT	GAT	GAA	ATG	GGA	918
Val	Lys	Gly	Leu	His	Phe	Ser	Gln	Glu	Asp	Ala	Pro	Asp	Glu	Met	Gly	
				285					290					295		
AAT	TAT	ATA	AAA	TCG	TTT	GTT	GAG	CGT	GTT	CTT	AAA	AAT	GAA	CAA	TAAACT	969
Asn	Tyr	Ile	Lys	Ser	Phe	Val	Glu	Arg	Val	Leu	Lys	Asn	Glu	Gln		

-29/43-

300	305	310	
ACCAGGTTTC	CATGTTGCCA	CTTTAGCTGG	GTTTAATAAA
TTTCACTATC	AATTTGAACA		1029
ATTTACATT	AATTTTAACT	ATTAAAAAAT	TATGGACACA
GGGATTATAT	CAGATGATTA		1089
ATTTAGTTGG	GAACAATGAA	TACCGAATAT	TATGAATTCT
CTTTAGCTAT	TTATAATAAT		1149
CACATTCTTA	TGTAATAAAA	CTTTGTTTTA	ATAAATTAAT
GATTCAGAAA	AAAAAAAAAA		1209
AAAAAAAA			1217

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 311 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Met	Thr	Ser	Lys	Val	Tyr	Asp	Pro	Glu	Leu	Arg	Lys	Arg	Met	Ile	Thr
1				5				10					15		
Gly	Pro	Gln	Trp	Trp	Ala	Arg	Cys	Lys	Gln	Met	Asn	Val	Leu	Asp	Ser
		20						25				30			
Phe	Ile	Asn	Tyr	Tyr	Asp	Ser	Glu	Lys	His	Ala	Glu	Asn	Ala	Val	Ile
		35					40				45				
Phe	Leu	His	Gly	Asn	Ala	Ala	Ser	Ser	Tyr	Leu	Trp	Arg	His	Val	Val
	50				55				60						
Pro	His	Val	Glu	Pro	Val	Ala	Arg	Cys	Ile	Ile	Pro	Asp	Leu	Ile	Gly
65					70				75					80	
Met	Gly	Lys	Ser	Gly	Lys	Ser	Gly	Asn	Gly	Ser	Tyr	Arg	Leu	Leu	Asp
			85					90					95		
His	Tyr	Lys	Tyr	Leu	Thr	Glu	Trp	Phe	Lys	His	Leu	Asn	Leu	Pro	Lys
		100					105					110			
Lys	Ile	Ile	Phe	Val	Gly	His	Asp	Trp	Gly	Ala	Cys	Leu	Ala	Phe	His
		115					120				125				
Tyr	Cys	Tyr	Glu	His	Gln	Asp	Arg	Ile	Lys	Ala	Val	Val	His	Ala	Glu
	130				135				140						
Ser	Val	Val	Asp	Val	Ile	Glu	Ser	Trp	Asp	Glu	Trp	Pro	Asp	Ile	Glu
145					150				155					160	
Glu	Asp	Ile	Ala	Leu	Ile	Lys	Ser	Glu	Glu	Gly	Glu	Lys	Met	Val	Leu
			165					170					175		
Glu	Asn	Asn	Phe	Phe	Val	Glu	Thr	Met	Leu	Pro	Ser	Lys	Ile	Met	Arg
		180					185					190			
Lys	Leu	Glu	Pro	Glu	Glu	Phe	Ala	Ala	Tyr	Leu	Glu	Pro	Phe	Lys	Glu
	195					200					205				
Lys	Gly	Glu	Val	Arg	Arg	Pro	Thr	Leu	Ser	Trp	Pro	Arg	Glu	Ile	Pro
	210				215						220				
Leu	Val	Lys	Gly	Gly	Lys	Pro	Asp	Val	Val	Glu	Ile	Val	Arg	Asn	Tyr
225					230				235					240	
Asn	Ala	Tyr	Leu	Arg	Ala	Ser	His	Asp	Leu	Pro	Lys	Met	Phe	Ile	Glu
			245					250					255		
Ser	Asp	Pro	Gly	Phe	Phe	Ser	Asn	Ala	Ile	Val	Glu	Gly	Ala	Lys	Lys
		260					265					270			
Phe	Pro	Asn	Thr	Glu	Phe	Val	Lys	Val	Lys	Gly	Leu	His	Phe	Ser	Gln
	275					280					285				
Glu	Asp	Ala	Pro	Asp	Glu	Met	Gly	Asn	Tyr	Ile	Lys	Ser	Phe	Val	Glu

-30/43-

290 295 300
 Arg Val Leu Lys Asn Glu Gln
 305 310

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 765 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 37...594

(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GCACGAGGGT ACTCAAAGTA TCTTCTGGCA GGGAAA ATG GGA GTG AAA GTT CTT	54
Met Gly Val Lys Val Leu	
1 5	
TTT GCC CTT ATT TGT ATT GCT GTG GCC GAG GCC AAA CCA ACT GAA AAC	102
Phe Ala Leu Ile Cys Ile Ala Val Ala Glu Ala Lys Pro Thr Glu Asn	
10 15 20	
AAT GAA GAT TTC AAC ATT GTA GCT GTA GCT AGC AAC TTT GCT ACA ACG	150
Asn Glu Asp Phe Asn Ile Val Ala Val Ala Ser Asn Phe Ala Thr Thr	
25 30 35	
GAT CTC GAT GCT GAC CGT GGT AAA TTG CCC GGA AAA AAA TTA CCA CTT	198
Asp Leu Asp Ala Asp Arg Gly Lys Leu Pro Gly Lys Lys Leu Pro Leu	
40 45 50	
GAG GTA CTC AAA GAA ATG GAA GCC AAT GCT AGG AAA GCT GGC TGC ACT	246
Glu Val Leu Lys Glu Met Glu Ala Asn Ala Arg Lys Ala Gly Cys Thr	
55 60 65 70	
AGG GGA TGT CTG ATA TGC CTG TCA CAC ATC AAG TGT ACA CCC AAA ATG	294
Arg Gly Cys Leu Ile Cys Leu Ser His Ile Lys Cys Thr Pro Lys Met	
75 80 85	
AAG AAG TTT ATC CCA GGA AGA TGC CAC ACC TAT GAA GGA GAC AAA GAA	342
Lys Lys Phe Ile Pro Gly Arg Cys His Thr Tyr Glu Gly Asp Lys Glu	
90 95 100	
AGT GCA CAG GGA GGA ATA GGA GAG GCT ATT GTT GAC ATT CCT GAA ATT	390
Ser Ala Gln Gly Gly Ile Gly Glu Ala Ile Val Asp Ile Pro Glu Ile	
105 110 115	
CCT GGG TTT AAG GAT TTG GAA CCC ATG GAA CAA TTC ATT GCA CAA GTT	438
Pro Gly Phe Lys Asp Leu Glu Pro Met Glu Gln Phe Ile Ala Gln Val	
120 125 130	

-31/43-

GAC CTA TGT GTA GAC TGC ACA ACT GGA TGC CTC AAA GGT CTT GCC AAT	486
Asp Leu Cys Val Asp Cys Thr Thr Gly Cys Leu Lys Gly Leu Ala Asn	
135 140 145 150	
GTG CAA TGT TCT GAT TTA CTC AAG AAA TGG CTG CCA CAA AGA TGT GCA	534
Val Gln Cys Ser Asp Leu Leu Lys Lys Trp Leu Pro Gln Arg Cys Ala	
155 160 165	
ACT TTT GCT AGC AAA ATT CAA GGC CAA GTG GAC AAA ATA AAG GGT GCC	582
Thr Phe Ala Ser Lys Ile Gln Gly Gln Val Asp Lys Ile Lys Gly Ala	
170 175 180	
GGT GGT GAT TAA TCCTAATAGA ATACTGCATA ACTGGATGAT GATATACTAG CTTATT	640
Gly Gly Asp *	
185	
GCTCATAAAA TGGCCATTTT TTGTAACAAA TCGAGTCTAT GTAATTCAAA ATACCTAATT	700
AATTGTTAAT ACATATGTAA TTCCTATAAA TATAATTTAT GCAATCCAAA AAAAAAAAAA	760
AAAAA	765

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 186 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Met Gly Val Lys Val Leu Phe Ala Leu Ile Cys Ile Ala Val Ala Glu	
1 5 10 15	
Ala Lys Pro Thr Glu Asn Asn Glu Asp Phe Asn Ile Val Ala Val Ala	
20 25 30	
Ser Asn Phe Ala Thr Thr Asp Leu Asp Ala Asp Arg Gly Lys Leu Pro	
35 40 45	
Gly Lys Lys Leu Pro Leu Glu Val Leu Lys Glu Met Glu Ala Asn Ala	
50 55 60	
Arg Lys Ala Gly Cys Thr Arg Gly Cys Leu Ile Cys Leu Ser His Ile	
65 70 75 80	
Lys Cys Thr Pro Lys Met Lys Lys Phe Ile Pro Gly Arg Cys His Thr	
85 90 95	
Tyr Glu Gly Asp Lys Glu Ser Ala Gln Gly Gly Ile Gly Glu Ala Ile	
100 105 110	
Val Asp Ile Pro Glu Ile Pro Gly Phe Lys Asp Leu Glu Pro Met Glu	
115 120 125	
Gln Phe Ile Ala Gln Val Asp Leu Cys Val Asp Cys Thr Thr Gly Cys	
130 135 140	
Leu Lys Gly Leu Ala Asn Val Gln Cys Ser Asp Leu Leu Lys Lys Trp	
145 150 155 160	
Leu Pro Gln Arg Cys Ala Thr Phe Ala Ser Lys Ile Gln Gly Gln Val	
165 170 175	
Asp Lys Ile Lys Gly Ala Gly Gly Asp	
180 185	

-32/43-

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1146 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

- (A) NAME/KEY: CBD Coding Sequence

- (B) LOCATION: 1...1146

- (D) OTHER INFORMATION: DNA encoding a CBD-Gaussia luciferase fusion protein

(ix) FEATURE:

- (A) NAME/KEY: CBD Coding Sequence

- (B) LOCATION: 1...591

(ix) FEATURE:

- (A) NAME/KEY: Gaussia luciferase

- (B) LOCATION: 591-1146

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

ATG TCA GTT GAA TTT TAC AAC TCT AAC AAA TCA GCA CAA ACA AAC TCA	48
Met Ser Val Glu Phe Tyr Asn Ser Asn Lys Ser Ala Gln Thr Asn Ser	
1 5 10 15	
ATT ACA CCA ATA ATC AAA ATT ACT AAC ACA TCT GAC AGT GAT TTA AAT	96
Ile Thr Pro Ile Ile Lys Ile Thr Asn Thr Ser Asp Ser Asp Leu Asn	
20 25 30	
TTA AAT GAC GTA AAA GTT AGA TAT TAT TAC ACA AGT GAT GGT ACA CAA	144
Leu Asn Asp Val Lys Val Arg Tyr Tyr Tyr Thr Ser Asp Gly Thr Gln	
35 40 45	
GGA CAA ACT TTC TGG TGT GAC CAT GCT GGT GCA TTA TTA GGA AAT AGC	192
Gly Gln Thr Phe Trp Cys Asp His Ala Gly Ala Leu Leu Gly Asn Ser	
50 55 60	
TAT GTT GAT AAC ACT AGC AAA GTG ACA GCA AAC TTC GTT AAA GAA ACA	240
Tyr Val Asp Asn Thr Ser Lys Val Thr Ala Asn Phe Val Lys Glu Thr	
65 70 75 80	
GCA AGC CCA ACA TCA ACC TAT GAT ACA TAT GTT GAA TTT GGA TTT GCA	288
Ala Ser Pro Thr Ser Thr Tyr Asp Thr Tyr Val Glu Phe Gly Phe Ala	
85 90 95	
AGC GGA GCA GCT ACT CTT AAA AAA GGA CAA TTT ATA ACT ATT CAA GGA	336
Ser Gly Ala Ala Thr Leu Lys Lys Gly Gln Phe Ile Thr Ile Gln Gly	
100 105 110	
AGA ATA ACA AAA TCA GAC TGG TCA AAC TAC ACT CAA ACA AAT GAC TAT	384
Arg Ile Thr Lys Ser Asp Trp Ser Asn Tyr Thr Gln Thr Asn Asp Tyr	
115 120 125	
TCA TTT GAT GCA AGT AGT TCA ACA CCA GTT GTA AAT CCA AAA GTT ACA	432

-33/43-

Ser	Phe	Asp	Ala	Ser	Ser	Ser	Thr	Pro	Val	Val	Asn	Pro	Lys	Val	Thr	
130						135					140					
GGA	TAT	ATA	GGT	GGA	GCT	AAA	GTT	CTT	GGT	ACA	GCA	CCA	GGT	TCC	GCG	480
Gly	Tyr	Ile	Gly	Gly	Ala	Lys	Val	Leu	Gly	Thr	Ala	Pro	Gly	Ser	Ala	
145					150					155					160	
GGT	CTG	GTG	CCA	CGC	GGT	AGT	ACT	GCA	ATT	GGT	ATG	AAA	GAA	ACC	GCT	528
Gly	Leu	Val	Pro	Arg	Gly	Ser	Thr	Ala	Ile	Gly	Met	Lys	Glu	Thr	Ala	
				165					170					175		
GCT	GCT	AAA	TTC	GAA	CGC	CAG	CAC	ATG	GAC	AGC	CCA	GAT	CTG	GGT	ACC	576
Ala	Ala	Lys	Phe	Glu	Arg	Gln	His	Met	Asp	Ser	Pro	Asp	Leu	Gly	Thr	
			180					185					190			
GAT	GAC	GAC	GAC	AAG	ATG	GGA	GTG	AAA	GTT	CTT	TTT	GCC	CTT	ATT	TGT	624
Asp	Asp	Asp	Asp	Lys	Met	Gly	Val	Lys	Val	Leu	Phe	Ala	Leu	Ile	Cys	
		195				200						205				
ATT	GCT	GTG	GCC	GAG	GCC	AAA	CCA	ACT	GAA	AAC	AAT	GAA	GAT	TTC	AAC	672
Ile	Ala	Val	Ala	Glu	Ala	Lys	Pro	Thr	Glu	Asn	Asn	Glu	Asp	Phe	Asn	
	210					215					220					
ATT	GTA	GCT	GTA	GCT	AGC	AAC	TTT	GCT	ACA	ACG	GAT	CTC	GAT	GCT	GAC	720
Ile	Val	Ala	Val	Ala	Ser	Asn	Phe	Ala	Thr	Thr	Asp	Leu	Asp	Ala	Asp	
225					230					235					240	
CGT	GGT	AAA	TTG	CCC	GGA	AAA	AAA	TTA	CCA	CTT	GAG	GTA	CTC	AAA	GAA	768
Arg	Gly	Lys	Leu	Pro	Gly	Lys	Lys	Leu	Pro	Leu	Glu	Val	Leu	Lys	Glu	
				245					250					255		
ATG	GAA	GCC	AAT	GCT	AGG	AAA	GCT	GGC	TGC	ACT	AGG	GGA	TGT	CTG	ATA	816
Met	Glu	Ala	Asn	Ala	Arg	Lys	Ala	Gly	Cys	Thr	Arg	Gly	Cys	Leu	Ile	
			260					265					270			
TGC	CTG	TCA	CAC	ATC	AAG	TGT	ACA	CCC	AAA	ATG	AAG	AAG	TTT	ATC	CCA	864
Cys	Leu	Ser	His	Ile	Lys	Cys	Thr	Pro	Lys	Met	Lys	Lys	Phe	Ile	Pro	
		275					280					285				
GGA	AGA	TGC	CAC	ACC	TAT	GAA	GGA	GAC	AAA	GAA	AGT	GCA	CAG	GGA	GGA	912
Gly	Arg	Cys	His	Thr	Tyr	Glu	Gly	Asp	Lys	Glu	Ser	Ala	Gln	Gly	Gly	
	290					295					300					
ATA	GGA	GAG	GCT	ATT	GTT	GAC	ATT	CCT	GAA	ATT	CCT	GGG	TTT	AAG	GAT	960
Ile	Gly	Glu	Ala	Ile	Val	Asp	Ile	Pro	Glu	Ile	Pro	Gly	Phe	Lys	Asp	
305					310					315					320	
TTG	GAA	CCC	ATG	GAA	CAA	TTC	ATT	GCA	CAA	GTT	GAC	CTA	TGT	GTA	GAC	1008
Leu	Glu	Pro	Met	Glu	Gln	Phe	Ile	Ala	Gln	Val	Asp	Leu	Cys	Val	Asp	
				325					330					335		
TGC	ACA	ACT	GGA	TGC	CTC	AAA	GGT	CTT	GCC	AAT	GTG	CAA	TGT	TCT	GAT	1056
Cys	Thr	Thr	Gly	Cys	Leu	Lys	Gly	Leu	Ala	Asn	Val	Gln	Cys	Ser	Asp	
			340					345					350			
TTA	CTC	AAG	AAA	TGG	CTG	CCA	CAA	AGA	TGT	GCA	ACT	TTT	GCT	AGC	AAA	1104
Leu	Leu	Lys	Lys	Trp	Leu	Pro	Gln	Arg	Cys	Ala	Thr	Phe	Ala	Ser	Lys	
		355					360					365				
ATT	CAA	GGC	CAA	GTG	GAC	AAA	ATA	AAG	GGT	GCC	GGT	GGT	GAT			1146
Ile	Gln	Gly	Gln	Val	Asp	Lys	Ile	Lys	Gly	Ala	Gly	Gly	Asp			

-34/43-

370

375

380

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 382 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE: CBD-Gaussia luciferase fusion protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Met	Ser	Val	Glu	Phe	Tyr	Asn	Ser	Asn	Lys	Ser	Ala	Gln	Thr	Asn	Ser	1	5	10	15
Ile	Thr	Pro	Ile	Ile	Lys	Ile	Thr	Asn	Thr	Ser	Asp	Ser	Asp	Leu	Asn	20	25	30	
Leu	Asn	Asp	Val	Lys	Val	Arg	Tyr	Tyr	Tyr	Thr	Ser	Asp	Gly	Thr	Gln	35	40	45	
Gly	Gln	Thr	Phe	Trp	Cys	Asp	His	Ala	Gly	Ala	Leu	Leu	Gly	Asn	Ser	50	55	60	
Tyr	Val	Asp	Asn	Thr	Ser	Lys	Val	Thr	Ala	Asn	Phe	Val	Lys	Glu	Thr	65	70	75	80
Ala	Ser	Pro	Thr	Ser	Thr	Tyr	Asp	Thr	Tyr	Val	Glu	Phe	Gly	Phe	Ala	85	90	95	
Ser	Gly	Ala	Ala	Thr	Leu	Lys	Lys	Gly	Gln	Phe	Ile	Thr	Ile	Gln	Gly	100	105	110	
Arg	Ile	Thr	Lys	Ser	Asp	Trp	Ser	Asn	Tyr	Thr	Gln	Thr	Asn	Asp	Tyr	115	120	125	
Ser	Phe	Asp	Ala	Ser	Ser	Ser	Thr	Pro	Val	Val	Asn	Pro	Lys	Val	Thr	130	135	140	
Gly	Tyr	Ile	Gly	Gly	Ala	Lys	Val	Leu	Gly	Thr	Ala	Pro	Gly	Ser	Ala	145	150	155	160
Gly	Leu	Val	Pro	Arg	Gly	Ser	Thr	Ala	Ile	Gly	Met	Lys	Glu	Thr	Ala	165	170	175	
Ala	Ala	Lys	Phe	Glu	Arg	Gln	His	Met	Asp	Ser	Pro	Asp	Leu	Gly	Thr	180	185	190	
Asp	Asp	Asp	Asp	Lys	Met	Gly	Val	Lys	Val	Leu	Phe	Ala	Leu	Ile	Cys	195	200	205	
Ile	Ala	Val	Ala	Glu	Ala	Lys	Pro	Thr	Glu	Asn	Asn	Glu	Asp	Phe	Asn	210	215	220	
Ile	Val	Ala	Val	Ala	Ser	Asn	Phe	Ala	Thr	Thr	Asp	Leu	Asp	Ala	Asp	225	230	235	240
Arg	Gly	Lys	Leu	Pro	Gly	Lys	Lys	Leu	Pro	Leu	Glu	Val	Leu	Lys	Glu	245	250	255	
Met	Glu	Ala	Asn	Ala	Arg	Lys	Ala	Gly	Cys	Thr	Arg	Gly	Cys	Leu	Ile	260	265	270	
Cys	Leu	Ser	His	Ile	Lys	Cys	Thr	Pro	Lys	Met	Lys	Lys	Phe	Ile	Pro	275	280	285	
Gly	Arg	Cys	His	Thr	Tyr	Glu	Gly	Asp	Lys	Glu	Ser	Ala	Gln	Gly	Gly	290	295	300	
Ile	Gly	Glu	Ala	Ile	Val	Asp	Ile	Pro	Glu	Ile	Pro	Gly	Phe	Lys	Asp	305	310	315	320
Leu	Glu	Pro	Met	Glu	Gln	Phe	Ile	Ala	Gln	Val	Asp	Leu	Cys	Val	Asp	325	330	335	

-35/43-

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Cys Thr Thr Gly Cys Leu Lys Gly Leu Ala Asn Val Gln Cys Ser Asp
      340                      345                      350
Leu Leu Lys Lys Trp Leu Pro Gln Arg Cys Ala Thr Phe Ala Ser Lys
      355                      360                      365
Ile Gln Gly Gln Val Asp Lys Ile Lys Gly Ala Gly Gly Asp
      370                      375                      380

```

(2) INFORMATION FOR SEQ ID NO:23

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) NAME/KEY:

(B) LOCATION:

(D) OTHER INFORMATION: Amino-terminal *Renilla reniformis* GFP peptide (Identity of residue #5 unknown)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```

Met Asp Leu Ala Xaa Lys Leu Gly Leu Lys Glu Val Met Pro Thr Lys
 1          5          10          15
Ile Asn Leu Glu Gly Leu Val Gly Asp His Ala Phe Ser Met Glu Gly
      20          25          30
Val Gly Glu Gly Asn Ile Leu Glu Gly
      35          40

```

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```

Val Lys Ile Ser Val Thr Lys Gly Ala Pro Leu Pro Phe Ala Phe Asp Ile
 1          5          10          15
Val Ser Val Ala Phe
      20

```

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

-36/43-

- (A) LENGTH: 40 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE: N-terminal
- (vi) ORIGINAL SOURCE:

- (A) NAME/KEY:
- (B) LOCATION:
- (D) OTHER INFORMATION: *Renilla reniformis* GFP peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Ala	Tyr	Thr	Gly	Tyr	Pro	Glu	Glu	Ile	Ser	Asp	Tyr	Phe	Leu	Gln	Ser	Phe
1				5					10					15		
Pro	Glu	Gly	Phe	Thr	Tyr	Glu	Arg	Gly	Asn	Ile	Arg	Tyr	Gln	Asp	Gly	Gly
	20						25					30				
Thr	Ala	Ile	Val	Lys	Ser											
35					40											

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 12 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE: N-terminal
- (vi) ORIGINAL SOURCE:

- (A) NAME/KEY:
- (B) LOCATION:
- (D) OTHER INFORMATION: *Renilla reniformis* GFP peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Ile	Val	Gly	Met	Gln	Pro	Ser	Tyr	Glu	Ser	Met	Tyr
1				5				10			

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 81 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE: N-terminal

-37/43-

(vi) ORIGINAL SOURCE:

(A) NAME/KEY:
 (B) LOCATION:
 (D) OTHER INFORMATION: Carboxyl-terminal *Renilla reniformis* GFP
 peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Thr Asn Val Thr Ser Val Ile Gly Gln Ile Ile Ala Phe Lys Leu Gln
 1 5 10 15
 Thr Gly Lys His Phe Thr Tyr His Met Arg Thr Val Tyr Lys Ser Lys
 20 25 30
 Lys Pro Val Glu Thr Met Pro Leu Tyr His Phe Ile Gln His Arg Leu
 35 40 45
 Val Lys Thr Asn Val Asp Thr Ala Ser Gly Tyr Val Val Gln His Glu
 50 55 60
 Thr Ala Ile Ala Ala His Ser Thr Ile Lys Lys Ile Glu Gly Ala Ala
 65 70 75 80
 Arg

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 861 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE: Pleuromamma

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
 (B) LOCATION: 148...741
 (D) OTHER INFORMATION: Pleuromamma luciferase

(x) PUBLICATION INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CGGCACGAGA TTTTGTCTGT GGTGATTGGG ATTGTCTGTC TCTCAGGTCA AGCAGAAAGT 60
 TCGCTGAAAG GTGATTTCTG TAGTGATGTT TCCTTCTGGG ATGTGATCAA GTACAACACT 120
 GAGAGTCGAC AATGCTGTGA CACAAAA ATG CTT AGA AAT TGC GCT AGG AAG CAA 174
 Met Leu Arg Asn Cys Ala Arg Lys Gln
 1 5
 GAG CAA GTT TGC GCC GAT GTG ACC GAG ATG AAA TGC CAA GCA GTT GCT 222
 Glu Gln Val Cys Ala Asp Val Thr Glu Met Lys Cys Gln Ala Val Ala
 10 15 20 25
 TGG GCC GAC TGT GGA CCC AGA TTT GAT TCC ACT GGC AGG AAT AGA TGC 270

-38/43-

Trp	Ala	Asp	Cys	Gly	Pro	Arg	Phe	Asp	Ser	Thr	Gly	Arg	Asn	Arg	Cys		
				30					35					40			
CAA	GTT	CAA	TAC	AAG	GAC	TAC	GCG	TAC	AAG	TCC	TGC	GTG	GAA	GTT	GAT	318	
Gln	Val	Gln	Tyr	Lys	Asp	Tyr	Ala	Tyr	Lys	Ser	Cys	Val	Glu	Val	Asp		
			45					50					55				
TAC	ACT	GTA	CCG	CAC	AGG	AAG	CAA	GTT	CCA	GAG	TGC	AAA	CAA	GTC	ACT	366	
Tyr	Thr	Val	Pro	His	Arg	Lys	Gln	Val	Pro	Glu	Cys	Lys	Gln	Val	Thr		
		60					65					70					
AAA	GAT	AAC	TGC	GTT	ACT	GAT	TGG	GAA	GTT	GAC	GCC	AAT	GGC	AAC	AAG	414	
Lys	Asp	Asn	Cys	Val	Thr	Asp	Trp	Glu	Val	Asp	Ala	Asn	Gly	Asn	Lys		
	75					80					85						
GTT	TGG	GGT	GGT	ACC	GAG	AAA	TGC	ACT	CCT	GTC	ACT	TGG	GAA	GAA	TGT	462	
Val	Trp	Gly	Gly	Thr	Glu	Lys	Cys	Thr	Pro	Val	Thr	Trp	Glu	Glu	Cys		
	90				95				100						105		
AAT	ATC	GTG	GAG	AAA	GAT	GTA	GAT	TTT	CCA	ACT	GTC	AAG	ACG	GAA	TGC	510	
Asn	Ile	Val	Glu	Lys	Asp	Val	Asp	Phe	Pro	Thr	Val	Lys	Thr	Glu	Cys		
					110					115					120		
GGC	ATC	CTG	TCT	CAC	CTT	AAG	TAT	GCA	GAC	TTC	ATA	GAG	GGA	CCT	TCC	558	
Gly	Ile	Leu	Ser	His	Leu	Lys	Tyr	Ala	Asp	Phe	Ile	Glu	Gly	Pro	Ser		
			125					130					135				
CAC	TCT	TTG	TCT	ATG	AGA	ACC	AAT	TGT	CAG	GTC	AAG	AGT	TCA	TTG	GAC	606	
His	Ser	Leu	Ser	Met	Arg	Thr	Asn	Cys	Gln	Val	Lys	Ser	Ser	Leu	Asp		
			140					145					150				
TGC	CGG	CCT	GTT	AAG	ACC	AGG	AAG	TGT	GCA	ACG	GTC	GAG	TAC	CAC	GAA	654	
Cys	Arg	Pro	Val	Lys	Thr	Arg	Lys	Cys	Ala	Thr	Val	Glu	Tyr	His	Glu		
		155					160					165					
TGC	AGC	ATG	AAG	CCC	CAA	GAA	GAC	TGC	AGC	CCA	GTC	ACT	GTT	CAT	ATT	702	
Cys	Ser	Met	Lys	Pro	Gln	Glu	Asp	Cys	Ser	Pro	Val	Thr	Val	His	Ile		
	170				175					180					185		
CCT	GAC	CAG	GAG	AAA	GTT	CAC	CAG	AAG	AAG	TGC	CTC	ACA	TAA			744	
Pro	Asp	Gln	Glu	Lys	Val	His	Gln	Lys	Lys	Cys	Leu	Thr					
					190					195							
ATGTTATCAA	TTTGTAGCTCT	TACTAATT	TAA	AACATAATAA	ATATCACATC	GAAGCCCTTT	804										
ATTTTATAGA	AGTGTAATGC	TTGAATAAAT	CTAGTGAATA	AAAAAAAAAA	AAAAAAA		861										

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 198 amino acids
- (B) TYPE: polypeptide
- (C) STRANDEDNESS: N/A
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

- (A) NAME/KEY: Pleuromamma luciferase
- (B) LOCATION:
- (D) OTHER INFORMATION: Pleuromamma luciferase

(vi) ORIGINAL SOURCE: Pleuromamma

(x) PUBLICATION INFORMATION

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

[illegible]

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1104 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE: *Ptilosarcus gurneyi*

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence (Insert A)

-40/43-

(B) LOCATION: 34...747

(D) OTHER INFORMATION: Ptilosarcus Green Fluorescent Protein
(GFP)

(x) PUBLICATION INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TCGGCACGAG CTGGCCTCCA CACTTTAGAC AAA ATG AAC CGC AAC GTA TTA AAG	54
Met Asn Arg Asn Val Leu Lys	
1 5	
AAC ACT GGA CTG AAA GAG ATT ATG TCG GCA AAA GCT AGC GTT GAA GGA	102
Asn Thr Gly Leu Lys Glu Ile Met Ser Ala Lys Ala Ser Val Glu Gly	
10 15 20	
ATC GTG AAC AAT CAC GTT TTT TCC ATG GAA GGA TTT GGA AAA GGC AAT	150
Ile Val Asn Asn His Val Phe Ser Met Glu Gly Phe Gly Lys Gly Asn	
25 30 35	
GTA TTA TTT GGA AAC CAA TTG ATG CAA ATC CGG GTT ACA AAG GGA GGT	198
Val Leu Phe Gly Asn Gln Leu Met Gln Ile Arg Val Thr Lys Gly Gly	
40 45 50 55	
CCG TTG CCA TTC GCT TTC GAT ATT GTT TCC ATA GCT TTC CAA TAC GGG	246
Pro Leu Pro Phe Ala Phe Asp Ile Val Ser Ile Ala Phe Gln Tyr Gly	
60 65 70	
AAT CGC ACT TTC ACG AAA TAC CCA GAC GAC ATT GCG GAC TAC TTT GTT	294
Asn Arg Thr Phe Thr Lys Tyr Pro Asp Asp Ile Ala Asp Tyr Phe Val	
75 80 85	
CAA TCA TTC CCG GCT GGA TTT TTC TAC GAA AGA AAT CTA CGC TTT GAA	342
Gln Ser Phe Pro Ala Gly Phe Phe Tyr Glu Arg Asn Leu Arg Phe Glu	
90 95 100	
GAT GGC GCC ATT GTT GAC ATT CGT TCA GAT ATA AGT TTA GAA GAT GAT	390
Asp Gly Ala Ile Val Asp Ile Arg Ser Asp Ile Ser Leu Glu Asp Asp	
105 110 115	
AAG TTC CAC TAC AAA GTG GAG TAT AGA GGC AAC GGT TTC CCT AGT AAC	438
Lys Phe His Tyr Lys Val Glu Tyr Arg Gly Asn Gly Phe Pro Ser Asn	
120 125 130 135	
GGA CCC GTG ATG CAA AAA GCC ATC CTC GGC ATG GAG CCA TCG TTT GAG	486
Gly Pro Val Met Gln Lys Ala Ile Leu Gly Met Glu Pro Ser Phe Glu	
140 145 150	
GTG GTC TAC ATG AAC AGC GGC GTT CTG GTG GGC GAA GTA GAT CTC GTT	534
Val Val Tyr Met Asn Ser Gly Val Leu Val Gly Glu Val Asp Leu Val	
155 160 165	
TAC AAA CTC GAG TCA GGG AAC TAT TAC TCG TGC CAC ATG AAA ACG TTT	582
Tyr Lys Leu Glu Ser Gly Asn Tyr Tyr Ser Cys His Met Lys Thr Phe	
170 175 180	
TAC AGA TCC AAA GGT GGA GTG AAA GAA TTC CCG GAA TAT CAC TTT ATC	630
Tyr Arg Ser Lys Gly Gly Val Lys Glu Phe Pro Glu Tyr His Phe Ile	
185 190 195	

-41/43-

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CAT CAT CGT CTG GAG AAA ACC TAC GTG GAA GAA GGA AGC TTC GTG GAA 678
His His Arg Leu Glu Lys Thr Tyr Val Glu Glu Gly Ser Phe Val Glu
200                205                210                215

CAA CAC GAG ACG GCC ATT GCA CAA CTG ACC ACA ATT GGA AAA CCT CTG 726
Gln His Glu Thr Ala Ile Ala Gln Leu Thr Thr Ile Gly Lys Pro Leu
                220                225                230

GGC TCC CTT CAT GAA TGG GTG TAG AAAATGACCA ATATACTGGG GAAACCGATA 780
Gly Ser Leu His Glu Trp Val
                235

ACCGTTTGGG AGCTTGTGTA TACAAATTAT TTGGGGTCAT TTTGTAATGT GTATGTGTGT 840
TGTATGATCA ATAGACGTCG TCATTCATAG CTTGAATCCT TCAGCAAAAG AAACCTCGAA 900
GCATATTGAA ACCTCGAAGC ATATTGAAAC CTCGACGGAG AGCGTAAAGA GACCGCACAA 960
ATTAACGCGT TTCAACCAGC AGTTGGAATC TTAAACCGA TCAAACTAT TAATATAAAT 1020
ATATATACCC TGTATAACTT ATATATATCT ATATAGTTTG ATATTGATTA AATCTGTTCT 1080
TGATCAAAAA AAAAAAAAAA AAAA 1104

```

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1279 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE: *Ptilosarcus gurneyi*

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence (Insert B)
- (B) LOCATION: 7...720
- (D) OTHER INFORMATION: *Ptilosarcus* Green Fluorescent Protein (GFP)

(x) PUBLICATION INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

```

GACAAA ATG AAC CGC AAC GTA TTA AAG 27
      Met Asn Arg Asn Val Leu Lys
          1                      5

AAC ACT GGA CTG AAA GAG ATT ATG TCG GCA AAA GCT AGC GTT GAA GGA 75
Asn Thr Gly Leu Lys Glu Ile Met Ser Ala Lys Ala Ser Val Glu Gly
          10                15                20

ATC GTG AAC AAT CAC GTT TTT TCC ATG GAA GGA TTT GGA AAA GGC AAT 123
Ile Val Asn Asn His Val Phe Ser Met Glu Gly Phe Gly Lys Gly Asn
          25                30                35

GTA TTA TTT GGA AAC CAA TTG ATG CAA ATC CGG GTT ACA AAG GGA GGT 171
Val Leu Phe Gly Asn Gln Leu Met Gln Ile Arg Val Thr Lys Gly Gly
          40                45                50                55

CCG TTG CCA TTC GCT TTC GAC ATT GTT TCC ATA GCT TTC CAA TAC GGG 219

```

-42/43-

Pro	Leu	Pro	Phe	Ala	Phe	Asp	Ile	Val	Ser	Ile	Ala	Phe	Gln	Tyr	Gly	
				60					65					70		
AAT	CGC	ACT	TTC	ACG	AAA	TAC	CCA	GAC	GAC	ATT	GCG	GAC	TAC	TTT	GTT	267
Asn	Arg	Thr	Phe	Thr	Lys	Tyr	Pro	Asp	Asp	Ile	Ala	Asp	Tyr	Phe	Val	
			75					80					85			
CAA	TCA	TTT	CCG	GCT	GGA	TTT	TTC	TAC	GAA	AGA	AAT	CTA	CGC	TTT	GAA	315
Gln	Ser	Phe	Pro	Ala	Gly	Phe	Phe	Tyr	Glu	Arg	Asn	Leu	Arg	Phe	Glu	
		90					95					100				
GAT	GGC	GCC	ATT	GTT	GAC	ATT	CGT	TCA	GAT	ATA	AGT	TTA	GAA	GAT	GAT	363
Asp	Gly	Ala	Ile	Val	Asp	Ile	Arg	Ser	Asp	Ile	Ser	Leu	Glu	Asp	Asp	
	105					110					115					
AAG	TTC	CAC	TAC	AAA	GTG	GAG	TAT	AGA	GGC	AAC	GGT	TTC	CCT	AGT	AAC	411
Lys	Phe	His	Tyr	Lys	Val	Glu	Tyr	Arg	Gly	Asn	Gly	Phe	Pro	Ser	Asn	
	120				125					130					135	
GGA	CCC	GTG	ATG	CAA	AAA	GCC	ATC	CTC	GGC	ATG	GAG	CCA	TCG	TTT	GAG	459
Gly	Pro	Val	Met	Gln	Lys	Ala	Ile	Leu	Gly	Met	Glu	Pro	Ser	Phe	Glu	
				140					145					150		
GTG	GTC	TAC	ATG	AAC	AGC	GGC	GTT	CTG	GTG	GGC	GAA	GTA	GAT	CTC	GTT	507
Val	Val	Tyr	Met	Asn	Ser	Gly	Val	Leu	Val	Gly	Glu	Val	Asp	Leu	Val	
			155					160					165			
TAC	AAA	CTC	GAG	TCA	GGG	AAC	TAT	TAC	TCG	TGC	CAC	ATG	AAA	ACG	TTT	555
Tyr	Lys	Leu	Glu	Ser	Gly	Asn	Tyr	Tyr	Ser	Cys	His	Met	Lys	Thr	Phe	
		170					175					180				
TAC	AGA	TCC	AAA	GGT	GGA	GTG	AAA	GAA	TTC	CCG	GAA	TAT	CAC	TTT	ATC	603
Tyr	Arg	Ser	Lys	Gly	Gly	Val	Lys	Glu	Phe	Pro	Glu	Tyr	His	Phe	Ile	
	185					190					195					
CAT	CAT	CGT	CTG	GAG	AAA	ACC	TAC	GTG	GAA	GAA	GGA	AGC	TTC	GTG	GAA	651
His	His	Arg	Leu	Glu	Lys	Thr	Tyr	Val	Glu	Glu	Gly	Ser	Phe	Val	Glu	
	200				205					210				215		
CAA	CAC	GAG	ACG	GCC	ATT	GCA	CAA	CTG	ACC	ACA	ATT	GGA	AAA	CCT	CTG	699
Gln	His	Glu	Thr	Ala	Ile	Ala	Gln	Leu	Thr	Thr	Ile	Gly	Lys	Pro	Leu	
				220				225						230		
GGC	TCC	CTT	CAT	GAA	TGG	GTG	TAG	AAAATGACCA	ATATACTGGG	GAAAATCACC						753
Gly	Ser	Leu	His	Glu	Trp	Val										
AATATACTGG	GGAAAATGAC	CAATTTACTG	GGGAAAATGA	CCAATATACT	GTAGAAAATC											813
ACCAATATAC	TGGGGAAAAT	GACCAATTTA	CTGGGGAAAT	GACCAATTTA	CTGTAGAAAA											873
TCACCAATAT	ACTGTGGAAA	ATGACCAAAA	TACTGTAGAA	ATGTTACACAC	TGGGTTGATA											933
ACCGTTTCGA	TAACCGTTTG	GAAGCTTGTG	TATACAAGTT	ATTTGGGGTC	ATTTTGTAAT											993
GTGTATGTGT	GTTGTATGAT	CTATAGACGT	CGTCATTCAT	AGCTTGAATC	CTTCAGCAAA											1053
AGAAACCTCG	AAGCATATTG	AAACCTCGAC	GGAGAGCATA	AAGAGACCGC	ACGTACACAA											1113
ATTATAATAC	CAGCAGTTGG	AATCTTTAAA	CCGATCAAAA	CTATTAATAT	ATATATACAC											1173
CCTGTATAAC	ATATATATAT	ATATATATCT	ACATAGTTTG	ATATTGATTA	AATCTGTTCT											1233
TGATCACTAA	AAAAAAAAAA	AAAAAAAAAA	AAAAAAAAAA	AAAAAA												1279

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 238 amino acids

-43/43-

(B) TYPE: polypeptide
 (C) STRANDEDNESS: N/A
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: Protein

(A) NAME/KEY: Ptilosarcus gurneyi Green Fluorescent Protein (GFP)
 (B) LOCATION:
 (D) OTHER INFORMATION: Ptilosarcus Green Fluorescent Protein (GFP)

(vi) ORIGINAL SOURCE: Ptilosarcus gurneyi

(x) PUBLICATION INFORMATION

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

```

Met Asn Arg Asn Val Leu Lys Asn Thr Gly Leu Lys Glu Ile Met Ser
1           5           10           15
Ala Lys Ala Ser Val Glu Gly Ile Val Asn Asn His Val Phe Ser Met
          20           25           30
Glu Gly Phe Gly Lys Gly Asn Asn Val Leu Phe Gly Asn Gln Leu Met Gln
          35           40           45
Ile Arg Val Thr Lys Gly Gly Pro Leu Pro Phe Ala Phe Asp Ile Val
          50           55           60
Ser Ile Ala Phe Gln Tyr Gly Asn Arg Thr Phe Thr Lys Tyr Pro Asp
65           70           75           80
Asp Ile Ala Asp Tyr Phe Val Gln Ser Phe Pro Ala Gly Phe Phe Tyr
          85           90           95
Glu Arg Asn Leu Arg Phe Glu Asp Gly Ala Ile Val Asp Ile Arg Ser
          100          105          110
Asp Ile Ser Leu Glu Asp Asp Lys Phe His Tyr Lys Val Glu Tyr Arg
          115          120          125
Gly Asn Gly Phe Pro Ser Asn Gly Pro Val Met Gln Lys Ala Ile Leu
          130          135          140
Gly Met Glu Pro Ser Phe Glu Val Val Tyr Met Asn Ser Gly Val Leu
145          150          155          160
Val Gly Glu Val Asp Leu Val Tyr Lys Leu Glu Ser Gly Asn Tyr Tyr
          165          170          175
Ser Cys His Met Lys Thr Phe Tyr Arg Ser Lys Gly Gly Val Lys Glu
          180          185          190
Phe Pro Glu Tyr His Phe Ile His His Arg Leu Glu Lys Thr Tyr Val
          195          200          205
Glu Glu Gly Ser Phe Val Glu Gln His Glu Thr Ala Ile Ala Gln Leu
          210          215          220
Thr Thr Ile Gly Lys Pro Leu Gly Ser Leu His Glu Trp Val
225          230

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 9/02, 15/54, 15/10, 15/62, C07K 16/28, A23L 1/03, A61K 7/00, F41C 3/00, C12G 1/00, 3/00, F21P 7/00		A3	(11) International Publication Number: WO 99/490
			(43) International Publication Date: 30 September 1999 (30.09.99)
(21) International Application Number: PCT/US99/06698			(72) Inventor; and
(22) International Filing Date: 26 March 1999 (26.03.99)			(75) Inventor/Applicant (for US only): SZENT-GYORGY Christopher [US/US]; 719 Duncan Avenue, Pittsburgh, Pa. 15237 (US).
(30) Priority Data:			(74) Agent: SEIDMAN, Stephanie, L.; Heller Ehrman White & McAuliffe, Suite 700, 4250 Executive Square, La Jolla, CA 92037 (US).
60/079,624 27 March 1998 (27.03.98) US			(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
60/089,367 15 June 1998 (15.06.98) US			
60/102,939 1 October 1998 (01.10.98) US			
(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications			
US 60/079,624 (CIP) Filed on 27 March 1998 (27.03.98) US 60/089,367 (CIP) Filed on 15 June 1998 (15.06.98) US 60/102,939 (CIP) Filed on 1 October 1998 (01.10.98)			
(71) Applicant (for all designated States except US): PROLUME, LTD. [US/US]; 1085 William Pitt Way, Pittsburgh, PA 15236 (US).			Published With international search report.
(71)(72) Applicant and Inventor: BRYAN, Bruce, J. [US/US]; 716 N. Arden Drive, Beverly Hills, CA 90210 (US).			
(88) Date of publication of the international search report: 29 June 2000 (29.06.00)			
(54) Title: LUCIFERASES, FLUORESCENT PROTEINS, NUCLEIC ACIDS ENCODING THE LUCIFERASES AND FLUORESCENT PROTEINS AND THE USE THEREOF IN DIAGNOSTICS			
(57) Abstract			
Isolated and purified nucleic acid molecules that encode a luciferase from <i>Renilla mulleri</i> , <i>Gaussia</i> and <i>Pleuromamma</i> , and the proteins encoded thereby are provided. Isolated and purified nucleic acids encoding green fluorescent proteins from the genus <i>Renilla</i> and <i>Pyrosoma</i> , and the green fluorescent proteins encoded thereby are also provided. Compositions and combinations comprising the green fluorescent proteins and/or the luciferase are further provided.			

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CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

INTERNATIONAL SEARCH REPORT

Int lional Application No
PCT/US 99/06698

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N9/02 C12N15/54 C12N15/10 C12N15/62 C07K16/28
A23L1/03 A61K7/00 F41C3/00 C12G1/00 C12G3/00
F21P7/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 29319 A (BRYAN BRUCE J) 14 August 1997 (1997-08-14) cited in the application abstract page 12, line 1 -page 16, line 27 page 26, line 5 -page 30, line 16 page 57, line 1 -page 130, line 15 SEQ.ID.N.1	1, 3, 5-11, 13-19, 22-31
X	US 5 418 155 A (CORMIER MILTON J ET AL) 23 May 1995 (1995-05-23) SEQ.ID.N.1 --- -/--	1, 3, 5-11

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

17 January 2000

Date of mailing of the international search report

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Authorized officer

Mateo Rosell, A.M.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/06698

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 15673 A (UNIV GEORGIA) 17 September 1992 (1992-09-17) the whole document and specially Fig.1 and 2 ---	1,3, 5-11, 13-19
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X	WO 95 18853 A (PROMEGA CORP) 13 July 1995 (1995-07-13) abstract page 9, line 5-13,29-35 page 12, line 25-35; table III ---	1
X	WO 95 25798 A (SECR DEFENCE BRITAIN) 28 September 1995 (1995-09-28) abstract page 2, paragraph 2 -page 3, last paragraph; example 10 ---	1
X	WO 97 11094 A (NOVONORDISK AS) 27 March 1997 (1997-03-27) abstract page 2, line 12 -page 5, line 9 ---	1
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X	EHRIG T ET AL: "GREEN-FLUORESCENT PROTEIN MUTANTS WITH ALTERED FLUORENCE EXCITATIONSPECTRA" FEBS LETTERS, vol. 367, 1 January 1995 (1995-01-01), pages 163-166, XP000579119 ISSN: 0014-5793 abstract --- -/--	1

INTERNATIONAL SEARCH REPORT

Int .tional Application No

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X	DATABASE EMBL NUCLEOTIDE AND PROTEIN SEQUENCES, 6 November 1997 (1997-11-06), XP002127788 HINXTON, GB AC= AF025844. Co-reporter vector pRL-Null, complete sequence. abstract ---	1,3, 5-11,72
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X	MITRA R D ET AL: "FLUORESCENCE RESONANCE ENERGY TRANSFER BETWEEN BLUE-EMITTING AND RED-SHIFTED EXCITATION DERIVATIVES OF THE GREEN FLUORESCENT PROTEIN" GENE, vol. 173, no. 1, 1 July 1996 (1996-07-01), pages 13-17, XP002033688 ISSN: 0378-1119 the whole document ---	72
X	HEIM R ET AL: "ENGINEERING GREEN FLUORESCENT PROTEIN FOR IMPROVED BRIGHTNESS, LONGER WAVELENGTHS AND FLUROESCENCE RESONANCE ENERGY TRANSFER" CURRENT BIOLOGY, vol. 6, no. 2, 1 February 1996 (1996-02-01), pages 178-182, XP000676582 ISSN: 0960-9822 the whole document --- -/--	72

INTERNATIONAL SEARCH REPORT

Int. l. Application No
PCT/US 99/06698

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	<p style="text-align: center;">---</p> <p>WARD W W ET AL: "AN ENERGY TRANSFER PROTEIN IN COELENTERATE BIOLUMINESCENCE CHARACTERIZATION OF THE RENILLA GREEN-FLUORESCENT PROTEIN"</p> <p>JOURNAL OF BIOLOGICAL CHEMISTRY (MICROFILMS), vol. 254, no. 3, 10 February 1979 (1979-02-10), pages 781-788, XP002030243 cited in the application the whole document</p>	1
A	<p style="text-align: center;">---</p> <p>DATABASE EMBL NUCLEOTIDE AND PROTEIN SEQUENCES, 18 February 1995 (1995-02-18), XP002127787 HINXTON, GB AC= U19278. Green fluorescent protein; cloning vector pGFP-C3. Clontech Laboratories, Inc. Unpublished. abstract</p>	32-43
A	<p style="text-align: center;">---</p> <p>SPURLOK B.O. ET AL.,: "A fine structure study of the anthocodium in Renilla mulleri"</p> <p>THE JOURNAL OF CELL BIOLOGY, vol. 64, 1975, pages 15-28, XP002118036 page 15-16 page 25 page 27</p>	1
A	<p style="text-align: center;">---</p> <p>LATZ M I ET AL., : "RECOVERY AND STIMULATION OF COPEPOD BIOLUMINESCENCE"</p> <p>JOURNAL OF EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY, vol. 136 (1), 1990, page 1-22 XP000864343 see discussion page 2, paragraph 4 -page 3, paragraph 2; table V</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	1

INTERNATIONAL SEARCH REPORT

Int lional Application No
PCT/US 99/06698

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
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A	SHIMOMURA O AND JOHNSON F H: "COMPARISON OF THE AMOUNTS OF KEY COMPONENTS IN THE BIO LUMINESCENCE SYSTEMS OF VARIOUS COELENTERATES" COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY B COMPARATIVE BIOCHEMISTRY, vol. 64 (1), 1979, page 105-108 XP000864339 figure 1	1
A	FRATAMICO P M ET AL.,: "Construction and characterization of Escherichia coli 0157:H7 strains expressing firefly luciferase and green fluorescent protein and their use in survival studies" JOURNAL OF FOOD PROTECTION, vol. 60, no. 10, 1997, pages 1167-1173, XP002118033 abstract page 1168, left-hand column, paragraphs 3,4 page 1169, left-hand column, paragraph 3 last paragraph from the discussion.	1,10,18, 29,30
A	SANDALOVA T. "Some notions about structure of bacterial luciferase, obtained by analysis of amino acid sequence, and study of monoclonal antibodies binding", IN: BIOLOGICAL LUMINESCENCE, PROCEEDINGS OF INTERNATIONAL SCHOOL,1st, (1990), 330-340. Ed., JEZOWSKA-TRZEBIATOWSKA et al., World Science, Sin XP002118038 the whole document	1,67,68
P,X	WO 98 14605 A (UNIV LOMA LINDA) 9 April 1998 (1998-04-09) page 2, line 9 -page 4, line 5 SEQ.ID.N.2	1,3, 5-11,67, 68,72
P,X	XU Y ET AL.,: "A bioluminescence resonance energy transfer (BRET) system: application to interacting circadian clock proteins" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 96, no. 1, 5 January 1999 (1999-01-05), pages 151-156, XP002118035 abstract materials and methods	72

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/06698

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1,3-11,13-19, 22-31,67,68,73,
75 partially and 72 completely

An isolated nucleic acid fragment, comprising a sequence of nucleotides encoding a *Renilla mullieri* luciferase (SEQ.ID.N.17); its corresponding amino acid sequence (SEQ.ID.N.18); vectors and hosts for the production of the recombinant protein; a bioluminescence generating system comprising luciferase, and a nucleic acid construct, comprising a nucleotide sequence encoding a luciferase and a green fluorescent protein.

2. Claims: 1,3-11,13-19,22-31,67,68,73,75 partially and 2,
72 and 74 completely

An isolated nucleic acid fragment, comprising a sequence of nucleotides encoding a *Gaussia princeps* luciferase (SEQ.ID.N.19); its corresponding amino acid sequence (SEQ.ID.N.20); vectors and hosts for the production of the recombinant protein; a bioluminescence generating system comprising luciferase, and a nucleic acid construct, comprising a nucleotide sequence encoding a luciferase and a green fluorescent protein.

3. Claims: 1,3-11,13-19,22-31,67,68,73,
75 partially and 72 completely

An isolated nucleic acid fragment, comprising a sequence of nucleotides encoding a *Pleuromamma* luciferase (SEQ.ID.N.26), its corresponding amino acid sequence (SEQ.ID.N.27); vectors and hosts for the production of the recombinant protein; a bioluminescence generating system comprising luciferase and a nucleic acid construct, comprising a nucleotide sequence encoding a luciferase and a green fluorescent protein.

4. Claims: 33-35 completely and 12,20,21,32,36-66,69-73,
75-99 partially

An isolated nucleic acid fragment, comprising a sequence of nucleotides encoding a *Renilla* green fluorescent protein (SEQ.ID.N.15); its corresponding amino acid sequence (SEQ.ID.N.16); vectors and hosts for the production of the recombinant protein; a bioluminescence generating system comprising luciferase and a nucleic acid construct, comprising a nucleotide sequence encoding a luciferase and a

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

green fluorescent protein.

5. Claims: 20,21,32,34-66,69-72,76-99 all partially

An isolated nucleic acid fragment, comprising a sequence of nucleotides encoding a Ptilosarcus green fluorescent protein (SEQ.ID.N.30,31); its corresponding amino acid sequence (SEQ.ID.N.32); vectors and hosts for the production of the recombinant protein; a bioluminescence generating system comprising luciferase and a nucleic acid construct, comprising a nucleotide sequence encoding a luciferase and a green fluorescent protein.

INTERNATIONAL SEARCH REPORT

information on patent family members

Int. Application No

PCT/US 99/06698

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9729319 A	14-08-1997	US 5876995 A AU 2255597 A CA 2245594 A EP 0879383 A JP 11504822 T	02-03-1999 28-08-1997 14-08-1997 25-11-1998 11-05-1999
US 5418155 A	23-05-1995	US 5292658 A	08-03-1994
WO 9215673 A	17-09-1992	DE 69131780 D EP 0575319 A JP 6504903 T	16-12-1999 29-12-1993 09-06-1994
WO 9518853 A	13-07-1995	AU 698424 B AU 1830395 A CA 2157476 A EP 0689587 A JP 8510387 T	29-10-1998 01-08-1995 13-07-1995 03-01-1996 05-11-1996
WO 9525798 A	28-09-1995	AU 697883 B AU 1954595 A BR 9507140 A CA 2186144 A CN 1149318 A EP 0751996 A FI 963741 A GB 2301592 A, B JP 9510610 T NO 963983 A	22-10-1998 09-10-1995 30-09-1997 28-09-1995 07-05-1997 08-01-1997 20-11-1996 11-12-1996 28-10-1997 23-09-1996
WO 9711094 A	27-03-1997	AT 184613 T AU 4482996 A CA 2232727 A DE 69604298 D EP 0851874 A JP 11512441 T	15-10-1999 09-04-1997 27-03-1997 21-10-1999 08-07-1998 26-10-1999
WO 9728261 A	07-08-1997	AU 2250497 A EP 0877805 A US 5981200 A	22-08-1997 18-11-1998 09-11-1999
WO 9814605 A	09-04-1998	US 5976796 A AU 4500497 A EP 0934425 A	02-11-1999 24-04-1998 11-08-1999

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(51) International Patent Classification 6 : C12N 9/02, 15/54, 15/10, 15/62, C07K 16/28, A23L 1/03, A61K 7/00, F41C 3/00, C12G 1/00, 3/00, F21S 10/00	A3	(11) International Publication Number: WO 99/49019 (43) International Publication Date: 30 September 1999 (30.09.99)
(21) International Application Number: PCT/US99/06698 (22) International Filing Date: 26 March 1999 (26.03.99) (30) Priority Data: 60/079,624 27 March 1998 (27.03.98) US 60/089,367 15 June 1998 (15.06.98) US 60/102,939 1 October 1998 (01.10.98) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications US 60/079,624 (CIP) Filed on 27 March 1998 (27.03.98) — US 60/089,367 (CIP) Filed on 15 June 1998 (15.06.98) US 60/102,939 (CIP) Filed on 1 October 1998 (01.10.98) (71) Applicant (for all designated States except US): PROLUME, LTD. [US/US]; 1085 William Pitt Way, Pittsburgh, PA 15236 (US). (71)(72) Applicant and Inventor: BRYAN, Bruce, J. [US/US]; 716 N. Arden Drive, Beverly Hills, CA 90210 (US).	(72) Inventor; and (75) Inventor/Applicant (for US only): SZENT-GYORGYI, Christopher [US/US]; 719 Duncan Avenue, Pittsburgh, PA 15237 (US). (74) Agent: SEIDMAN, Stephanie, L.; Heller Ehrman White & McAuliffe, Suite 700, 4250 Executive Square, La Jolla, CA 92037 (US). (81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With a revised version of the international search report.</i> (88) Date of publication of the international search report: 29 June 2000 (29.06.00) (88) Date of publication of the revised version of the international search report: 8 September 2000 (08.09.00)	
(54) Title: LUCIFERASES, FLUORESCENT PROTEINS, NUCLEIC ACIDS ENCODING THE LUCIFERASES AND FLUORESCENT PROTEINS AND THE USE THEREOF IN DIAGNOSTICS (57) Abstract Isolated and purified nucleic acid molecules that encode a luciferase from <i>Renilla mulleri</i> , <i>Gaussia</i> and <i>Pleuromamma</i> , and the proteins encoded thereby are provided. Isolated and purified nucleic acids encoding green fluorescent proteins from the genus <i>Renilla</i> and <i>Ptilosarcus</i> , and the green fluorescent proteins encoded thereby are also provided. Compositions and combinations comprising the green fluorescent proteins and/or the luciferase are further provided.		

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	F21S10/00				

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IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Date of the actual completion of the international search

19 June 2000

Date of mailing of the international search report

04. 2. 00

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INTERNATIONAL SEARCH REPORT

Internatic Application No

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ROMOSER V.A. ET AL.,: "Detection in living cells of Ca ²⁺ -dependent changes in the fluorescence emission of an indicator composed of two green fluorescent protein variants linked by a calmodulin-binding sequence." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 20, 16 May 1997 (1997-05-16), pages 13270-13274, XP002118032 materials and methods. abstract	72
A	--- WARD W W ET AL: "AN ENERGY TRANSFER PROTEIN IN COELENTERATE BIOLUMINESCENCE CHARACTERIZATION OF THE RENILLA GREEN-FLUORESCENT PROTEIN" JOURNAL OF BIOLOGICAL CHEMISTRY (MICROFILMS), vol. 254, no. 3, 10 February 1979 (1979-02-10), pages 781-788, XP002030243 cited in the application the whole document	1
A	--- DATABASE EMBL NUCLEOTIDE AND PROTEIN SEQUENCES, 18 February 1995 (1995-02-18), XP002127787 HINXTON, GB AC= U19278. Green fluorescent protein; cloning vector pGFP-C3. Clontech Laboratories, Inc. Unpublished. abstract	32-43
A	--- SPURLOK B.O. ET AL.,: "A fine structure study of the anthocodium in Renilla mulleri" THE JOURNAL OF CELL BIOLOGY, vol. 64, 1975, pages 15-28, XP002118036 page 15-16 page 25 page 27	1
A	--- LATZ M I ET AL.,: "RECOVERY AND STIMULATION OF COPEPOD BIOLUMINESCENCE" JOURNAL OF EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY, vol. 136 (1), 1990, page 1-22 XP000864343 see discussion page 2, paragraph 4 -page 3, paragraph 2; table V --- -/--	1

INTERNATIONAL SEARCH REPORT

Internatic Application No
PCT/US 99/06698

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SHIMOMURA O AND JOHNSON F H: "COMPARISON OF THE AMOUNTS OF KEY COMPONENTS IN THE BIO LUMINESCENCE SYSTEMS OF VARIOUS COELENTERATES" COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY B COMPARATIVE BIOCHEMISTRY, vol. 64 (1), 1979, page 105-108 XP000864339 figure 1	1
A	FRATAMICO P M ET AL.,: "Construction and characterization of Escherichia coli 0157:H7 strains expressing firefly luciferase and green fluorescent protein and their use in survival studies" JOURNAL OF FOOD PROTECTION, vol. 60, no. 10, 1997, pages 1167-1173, XP002118033 abstract page 1168, left-hand column, paragraphs 3,4 page 1169, left-hand column, paragraph 3 last paragraph from the discussion.	1,10,18, 29,30
A	SANDALOVA T. "Some notions about structure of bacterial luciferase, obtained by analysis of amino acid sequence, and study of monoclonal antibodies binding", IN: BIOLOGICAL LUMINESCENCE, PROCEEDINGS OF INTERNATIONAL SCHOOL,1st, (1990), 330-340. Ed., JEZOWSKA-TRZEBIATOWSKA et al., World Science, Sin XP002118038 the whole document	1,67,68
P,X	WO 98 14605 A (UNIV LOMA LINDA) 9 April 1998 (1998-04-09) page 2, line 9 -page 4, line 5 SEQ.ID.N.2	1,3, 5-11,67, 68,72
P,X	XU Y ET AL.,: "A bioluminescence resonance energy transfer (BRET) system: application to interacting circadian clock proteins" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 96, no. 1, 5 January 1999 (1999-01-05), pages 151-156, XP002118035 abstract materials and methods	72

INTERNATIONAL SEARCH REPORT

Inter. application No.
PCT/US 99/06698

Box I Observations where certain claims were found unsatisfactory (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1,3-11,13-19, 22-31,67,68,73,
75 partially and 72 completely

An isolated nucleic acid fragment, comprising a sequence of nucleotides encoding a Renilla mullieri luciferase (SEQ.ID.N.17); its corresponding amino acid sequence (SEQ.ID.N.18); vectors and hosts for the production of the recombinant protein; a bioluminescence generating system comprising luciferase, and a nucleic acid construct, comprising a nucleotide sequence encoding a luciferase and a green fluorescent protein.

2. Claims: 1,3-11,13-19,22-31,67,68,73,75 partially and 2,
72 and 74 completely

An isolated nucleic acid fragment, comprising a sequence of nucleotides encoding a Gaussia princeps luciferase (SEQ.ID.N.19); its corresponding amino acid sequence (SEQ.ID.N.20); vectors and hosts for the production of the recombinant protein; a bioluminescence generating system comprising luciferase, and a nucleic acid construct, comprising a nucleotide sequence encoding a luciferase and a green fluorescent protein.

3. Claims: 1,3-11,13-19,22-31,67,68,73,
75 partially and 72 completely

An isolated nucleic acid fragment, comprising a sequence of nucleotides encoding a Pleuromamma luciferase (SEQ.ID.N.26), its corresponding amino acid sequence (SEQ.ID.N.27); vectors and hosts for the production of the recombinant protein; a bioluminescence generating system comprising luciferase and a nucleic acid construct, comprising a nucleotide sequence encoding a luciferase and a green fluorescent protein.

4. Claims: 33-35 completely and 12,20,21,32,36-66,69-73,
75-99 partially

An isolated nucleic acid fragment, comprising a sequence of nucleotides encoding a Renilla green fluorescent protein (SEQ.ID.N.15); its corresponding amino acid sequence (SEQ.ID.N.16); vectors and hosts for the production of the recombinant protein; a bioluminescence generating system comprising luciferase and a nucleic acid construct, comprising a nucleotide sequence encoding a luciferase and a

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

green fluorescent protein.

5. Claims: 20,21,32,34-66,69-72,76-99 all partially

An isolated nucleic acid fragment, comprising a sequence of nucleotides encoding a Ptilosarcus green fluorescent protein (SEQ.ID.N.30,31); its corresponding amino acid sequence (SEQ.ID.N.32); vectors and hosts for the production of the recombinant protein; a bioluminescence generating system comprising luciferase and a nucleic acid construct, comprising a nucleotide sequence encoding a luciferase and a green fluorescent protein.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat. Application No

PCT/US 99/06698

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9729319 A	14-08-1997	US 5876995 A AU 2255597 A CA 2245594 A EP 0879383 A JP 11504822 T	02-03-1999 28-08-1997 14-08-1997 25-11-1998 11-05-1999
US 5418155 A	23-05-1995	US 5292658 A	08-03-1994
WO 9215673 A	17-09-1992	DE 69131780 D EP 0575319 A ES 2142801 T JP 6504903 T	16-12-1999 29-12-1993 01-05-2000 09-06-1994
WO 9518853 A	13-07-1995	AU 698424 B AU 1830395 A CA 2157476 A EP 0689587 A JP 8510387 T	29-10-1998 01-08-1995 13-07-1995 03-01-1996 05-11-1996
WO 9525798 A	28-09-1995	AU 697883 B AU 1954595 A BR 9507140 A CA 2186144 A CN 1149318 A EP 0751996 A FI 963741 A GB 2301592 A,B JP 9510610 T NO 963983 A	22-10-1998 09-10-1995 30-09-1997 28-09-1995 07-05-1997 08-01-1997 20-11-1996 11-12-1996 28-10-1997 23-09-1996
WO 9711094 A	27-03-1997	AT 184613 T AU 4482996 A CA 2232727 A DE 69604298 D DE 69604298 T EP 0851874 A ES 2139329 T JP 11512441 T	15-10-1999 09-04-1997 27-03-1997 21-10-1999 18-05-2000 08-07-1998 01-02-2000 26-10-1999
WO 9728261 A	07-08-1997	AU 717873 B AU 2250497 A EP 0877805 A US 5981200 A	06-04-2000 22-08-1997 18-11-1998 09-11-1999
WO 9814605 A	09-04-1998	US 5976796 A AU 4500497 A EP 0934425 A	02-11-1999 24-04-1998 11-08-1999

